**University of Alberta**

# The Influence of Commercial Probiotics and Breeder Flock Age on Broiler Chick Quality and Production Efficiency

by

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# DEDICATION

For Mom and Dad,

who have always believed that I was capable of anything.

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# LIST OF ABBREVIATIONS

ARDRA = amplified ribosomal DNA restriction analysis

ATCC = American type culture collection

BHI = Brain heart infusion

CFIA = Canadian Food Inspection Agency

CFU = colony forming unit

CON = control

d = days

FCR = feed conversion ratio

GIT = gastrointestinal tract

GLM = general linear model

h = hours

IBS = Interbac® spray

IBW = Interbac® water

LAB = lactic acid bacteria

LBS = *Lactobacillus* selective

ME = metabolizable energy

min = minutes

MRS = Man Rogosa Sharp

NE = necrotic enteritis

PAF = Pro-Avi® Feed

PCR = polymerase chain reaction

RAPD = random amplification of polymorphic DNA

RFLP = restriction fragment length polymorphism

VBS = Veterinary Biologics Section

wk = weeks

# 1. GENERAL INTRODUCTION

## THE CANADIAN BROILER INDUSTRY

The poultry industry in Canada has experienced steady growth over the past 15 years (Alberta Chicken Producers, 2004a). In 2002, the 2,851 broiler producers operating in Canada were responsible for the production of over 938 million kg of broiler chicken meat, most of which was consumed within Canada (Chicken Farmers of Canada, 2003).

Broilers in Canada are reared on straw or wood shavings in large, climate controlled barns with an open-floor design (Alberta Chicken Producers, 2004b). The birds have unlimited access to a grain based diet, and to fresh, clean water at all times during rearing (Alberta Chicken Producers, 2004b). The average broiler producer rears 6.5 flocks per year, with over 34,000 birds per flock (Chicken Farmers of Canada, 2003; Alberta Chicken Producers, 2004b). Between flocks, all manure and used litter is removed from the barns, and the barns cleaned and disinfected (Alberta Chicken Producers, 2004b).

Broilers are usually reared for a period of six wk, but may be reared for up to eight wk depending on the desired market weight (Alberta Chicken Producers, 2004b). Generally, a modern genetic strain of broiler reared for six wk will reach a live weight of approximately 2.0 to 2.5 kg (Cobb-Vantress Inc., 2003; Aviagen Inc., 2004). Since broiler producers in Canada are paid on the basis of kg of eviscerated carcass weight, factors that affect broiler growth and production efficiency are of the utmost importance.

# SELECTED FACTORS INFLUENCING THE PRODUCTION EFFICIENCY OF BROILERS

## The Effect of Breeder Flock Age

***Chick Quality.*** There are inconsistencies in the quality of chicks that broiler producers receive. Many factors contribute to this variation in quality, and thus affect performance characteristics and survivability (Sinclair *et al.*, 1990). One of the main factors affecting chick quality and performance is chick size, which is influenced by flock age, with younger flocks producing smaller eggs and smaller chicks. There is a strong correlation between egg weight and chick weight at hatching (O'Neil, 1955; McNaughton *et al.*, 1978; Wyatt *et al.*, 1985). The strength of this correlation weakens as the chick ages to six wk, but is still evident (Wiley, 1950; Goodwin, 1961; Tindell and Morris, 1964, Merritt and Gowe, 1965, Morris *et al.*, 1968) so the larger broiler chicks produced by older breeder flocks still have higher market body weights (Sklan *et al.*, 2003). The strength of this correlation also varies considerably with strain as broilers age (Kosin *et al.*, 1952; Pope and Schaible, 1957; Bray and Iton, 1962; Merritt and Gowe, 1965; Gupta and Johar, 1975).

Chick quality is also affected by eggshell conductance, and the total amount of moisture lost through the shell during incubation (Tullett and Burton, 1982). Since eggshell conductance increases with flock age (allowing for a higher rate of gas exchange), this is another factor that may result in poorer quality chicks from younger flocks.

***Broiler Mortality.*** Up to 60% of total broiler mortality occurs within the first wk of production, and this percentage can be greatly influenced by the age of the breeder flock producing the broiler chicks (McNaughton *et al*., 1978). Chicks from smaller eggs produced by younger flocks experience higher mortality than larger chicks from older breeder flocks (McClung and Smith, 1949; O'Neil, 1950; Wiley, 1950; Hays and Spear, 1952; Skoglund *et al*., 1952; McNaughton *et al.,* 1978; Wyatt *et al*., 1985; Hearn, 1986). Even when egg weight is equalized, mortality is higher in chicks from a young flock compared to an old flock (McNaughton *et al*., 1978). It has also been found that there is higher mortality in small eggs from a young flock compared to large eggs from a young flock and that when egg weight is removed as a factor, there is no difference in the market weights of broilers produced at different breeder flock ages (McNaughton *et al*., 1978). The increased viability in the chicks from older breeder flocks may be due to the fact that the chicks from older breeder flocks also have an increased percentage of body fat at hatch compared to chicks from a younger breeder flock (McNaughton *et al*., 1978).

***Feed Conversion.*** The research is not definitive with respect to whether feed conversion ratio (FCR) is different between small and large chicks. While some researchers have determined that chicks produced from large eggs have improved FCR over those chicks from small eggs (O'Neil 1950; Wiley, 1950; Proudfoot *et al*., 1982), others have found the opposite (O'Neil, 1955; Morris *et al*., 1968; Guill and Washburn, 1973; Proudfoot and Hulan, 1981; Wyatt *et al*., 1985; Hearn, 1986). It does appear that whatever the differences in chick weight, feed intake is the main factor influencing final market body weight (Shanawany, 1987).

Past research has evaluated the effect of egg size and post-hatch holding of chicks on early broiler mortality and performance (Fanguy *et al.*, 1980; Hager and Beane, 1983; Reinhart and Hurnik, 1984; Wyatt *et al.,* 1985). Post-hatch holding (the time between the time the chicks hatch and the time they are placed in the barn), which results in dehydration, has a greater impact on chicks from small eggs produced by younger flocks; the primary reason is the early hatching time of these chicks (Pinchasov and Noy, 1993).

## The Effect of Bacterial Infections in the Gastrointestinal Tract

***Salmonella.*** The *Salmonella* species most commonly found in poultry are of the paratyphoid type, including S. Enteritidis and S. Typhimurium. Paratyphoid *Salmonella* can cause disease in young chicks, but do little harm to mature birds. In most cases, birds infected with paratyphoid *Salmonella* will show no symptoms. The exception is for young chicks, which may be listless or experience diarrhea (Culter, 2002). However, the bacteria can survive for weeks in water, on food, and in the environment. These bacteria pose a threat to human health, causing salmonellosis if contaminated chicken meat is consumed (Culter, 2002). It is this hazard to food safety that makes *Salmonella* infection in poultry a major concern.

***Necrotic Enteritis.*** Necrotic enteritis (NE), on the other hand does influence bird health. This is a disease of poultry that results in depressed growth and high mortality (Culter, 2002). The causative agent of NE is the toxigenic bacterium *Clostridium perfringens* (Al-Sheikhly and Truscott, 1977). *C. perfringens* causes NE when the bacterium comes into contact with damaged portions of the lining of the small intestine. As such, NE is a common secondary infection that often follows *Salmonella* infections or coccidiosis outbreaks, when the intestinal lining is already irritated, or the immune system is suppressed (Culter, 2002), predisposing the bird to NE (Annett *et al.*, 2002). Feeding high levels of wheat is known to change the composition of the gastrointestinal tract (GIT) microflora in such a way that *C. perfringens* is able to grow out of control (Culter, 2002). The most common means to controlling the growth of *Clostridium perfringens*, and other unfavorable bacteria in the GIT is through the use of antibiotics at sub-therapeutic levels (Culter, 2002).

## THE GASTROINTESTINAL TRACT OF POULTRY

### The Oral Cavity

In poultry there is no distinction between where the mouth ends and the pharynx begins. Together this cavity is called the oropharynx (Denbow, 2000). The purpose of the tongue in poultry is to collect and manipulate feed. The area near the root of the tongue is covered with papillae, which propel feed down toward the esophagus (Denbow, 2000). The salivary glands are well developed in domestic poultry (Hill, 1976), and are located both in the roof and floor of the mouth, as well as on the sides of the mouth (Denbow, 2000). In domestic poultry, very little amylase is secreted by the salivary glands in the oral cavity (Jerrett and Goodge, 1973).

### The Esophagus and Crop

The esophagus is a simple tube that runs from the pharynx to the stomach, and lacks sphincters at either end (Denbow, 2000). It has very thin walls with longitudinal folds, to allow for expandability since the feed is swallowed whole. Peristalsis in the esophagus moves the feed bolus towards the proventriculus (Hill, 1976). The lining of the esophagus and crop is composed of partially keratinized, stratified squamous epithelial cells and mucous glands (Denbow, 2000).

In the chicken, the crop is basically an expansion of the cervical esophagus that acts as a temporary food storage organ. Since the stratified squamous epithelial cells lining the crop are non-secretory they are not covered in mucous (Mead, 1997). The crop contains mucous glands only at the junction with the esophagus (Denbow, 2000). The lining of the crop is deeply folded, allowing it to expand to accommodate temporary feed storage (Denbow, 2000). Food can remain stored in the crop for as long as 20 h (Mead, 1997). Food in the crop is passed on to the proventriculus via contraction of the crop wall (Hill, 1976).

## The Proventriculus

The proventriculus, or glandular stomach, in poultry species is the structure that is most comparable to the mammalian stomach. The proventriculus is a relatively small organ in domestic poultry. The inner surface of the proventriculus is and covered in a mucous membrane. The mucous membrane is covered in papillae, on the surface of which are openings of the compound glands, responsible for the secretion of gastric juices (Denbow, 2000). Contained in these glands are oxynticopeptic cells, responsible for the secretion of hydrochloric acid, pepsin, and mucous (Denbow, 2000).

## The Gizzard

The gizzard, or muscular stomach, is the site of mechanical digestion and is a relatively large and powerful organ designed for crushing. It is composed of two opposing pairs of muscles, called the thick (lateral thick and medial thick) and thin (cranial thin and caudal thin) pairs (Denbow, 2000). Each pair consists of circular muscle. The inner lining of the gizzard is composed of koilin, a type of cuticle secreted by the mucosal glands (Denbow, 2000). This serves a protective function, guarding the gizzard against damage by the acid and proteolytic enzymes secreted in the proventriculus. The koilin lining also protects the gizzard from damage due to mechanical grinding of feed (Denbow, 2000). The pyloric region of the gizzard connects the gizzard and duodenum. The pyloric region contains mucosal glands that secrete mucous, rather than koilin (Denbow, 2000). Retrograde movement of food can occur once the food bolus is in the duodenum; the duodenal muscles contract and the isthmus relaxes while the thick muscles of the gizzard contract. The material in the gizzard is then refluxed into the proventriculus. The cycle is completed when the proventriculus contracts and the bolus is forced back into the gizzard (Denbow, 2000).

## The Small Intestine

In the average broiler chicken the small intestine accounts for 82.7% of the total GIT weight (Denbow, 2000). It is the main site of nutrient absorption. Although all sections of the small intestine share similar histology, the small intestine can be divided into three sections: the duodenum, jejunum and ileum. There is a distinct structure called the duodenal loop that indicates the location of the duodenum, while the junction between the jejunum and ileum is identified by the diverticulum vitellinum, also called Meckel's diverticulum (Denbow, 2000). The villi lining the small intestine are approximately 1.5 mm high in the duodenum, and decrease in height throughout the small intestine to 0.4 to 0.6 mm in the ileum (Denbow, 2000). Throughout all three segments of the small intestine, the mucosa is comprised of columnar epithelial cells and goblet cells, responsible for secreting mucous (Hill, 1976).

The four layers present in the small intestine (mucosa, submucosa, muscle tunic and serosa) are the same four layers present elsewhere in the digestive tract (Denbow, 2000). The mucosa (innermost layer) is composed of the epithelium, the lamina propria, and the muscularis mucosa. Of these three components of the mucosa, the epithelium is the most developed in avian species. The epithelium has several types of cells with different functions (chief cells, goblet cells, and endocrine cells). The epithelial cells that line the villi in the GIT arise from the crypts of Liberkuhn (Denbow, 2000). The crypts also contain lymphocytes, goblet cells, endocrine cells and undifferentiated cells, with leukocytes and Paneth cells, which are present at the base of the crypts (Denbow, 2000). The villi are arranged in a zig-zag formation, which is thought to play a role in slowing the flow of digesta, thus increasing transit time to allow for better nutrient absorption (Denbow, 2000).

## The Ceca

Poultry species possess paired ceca, located at the junction of the small and large intestines. The ceca are sites of fermentation (Hill, 1976), detoxification of harmful substances, and may also be the site of small amounts of nutrient absorption (Gong *et al.*, 2002). However, in poultry, they do not appear to produce sufficient quantities of fermentation products to contribute significantly to the energy intake of the bird (Moran, 1982). Each cecum is divided into three regions: the basis ceci (near the ileocecal junction), the corpus ceci (medial cecal region), and the apex ceci (distal cecal region). The ceca are lined with columnar epithelial cells, and the morphology of the villi varies throughout the cecum. The basis ceci has very well developed villi, the corpus ceci has small villi and longitudinal folds in the walls, and the apex ceci has the same small villi as the corpus ceci, but there are transverse as well as longitudinal folds in the walls. The musculature and the well-developed villi near the ileocecal junction keep out all particulate matter, so only liquid enters the ceca (Denbow, 2000).

The antiperistaltic refluxing of uric acid into the ceca is not only important for water balance, but is also important in that it exposes the cecal microflora to urea and uric acid which are broken down to provide the microflora with a source of nitrogen. There is also some microbial synthesis of B complex vitamins by the bacterial species in the ceca. These vitamins are not absorbed by the host, but are instead secreted and expelled in the feces. The vitamins are then available to the bird only through coprophagy (Duke, 1993).

## The Large Intestine

The large intestine, also called the colon, connects the ileum to the coprodeal compartment of the cloaca. This region of the digestive tract has many flat villi and few goblet cells (Clauss *et al.*, 1991). The large intestine is short in length, and the flora is indistinguishable from that present in the ceca. This is as expected since there is a great deal of exchange of material between these organs (Hill, 1976). Antiperistalsis occurs in the large intestine of poultry and serves two main functions. The first function is to move uric acid from the cloaca into the large intestine and the ceca for water absorption (Denbow, 2000). The second is to fill the ceca with digesta for further nutrient absorption (Duke, 1993).

## THE COMMENSAL MICROFLORA

The commensal microflora is a term used to describe the populations of bacteria that normally reside in the GIT of a healthy bird. There are several criteria established by Savage (1977), which aid in differentiating members of the commensal microflora from transient microorganisms, unfavorable microorganisms, and pathogens in the GIT. In order for a bacterial species to be considered a member of the commensal microflora, it must always be found in stable populations in normal, healthy, mature animals of the species of interest and must be able to colonize particular areas of the GIT. This colonization should follow a succession of predominant species through the development of the animal. Members of the commensal microflora are often in close association with the mucosal epithelial cells and usually thrive in the lumen if nutrient availability is not limiting (Savage, 1977).

Interest in the composition of the chicken GIT microflora has been rapidly increasing over the past few decades. Researchers are striving to understand how the microorganisms present impact bird health and growth rate, and how these same microorganisms can be manipulated to improve production traits in commercial poultry. The effect of the microflora on the host can be either positive or negative, depending on its composition (Barrow, 1992). The microorganisms normally present in the GIT of poultry have the ability to competitively exclude pathogens, thus preventing disease and the associated growth depression. This is of particular interest as the poultry industry shifts its focus away from the use of prophylactic antibiotics and toward other means of improving growth and preventing disease.

Within a few hours of hatching, bacterial populations develop throughout the GIT of the chick (Guan *et al.*, 2004). The first exposure to bacteria post-hatch is thought to be through contact with bacteria on the exterior of the eggshell (Coates and Fuller, 1977) or through consumption of feed containing microorganisms (Smith, 1965; Barnes, 1972; Mead and Adams, 1975). Initially, the chick's GIT is more vulnerable to colonization by transient microorganisms, but the numbers of the transient microorganisms colonizing the GIT tend to decrease as more microbial competition is introduced over time (Bailey *et al.*, 1988). The colonization of the GIT is determined both by exposure to the maternal intestinal flora, and environmental exposure to various bacteria.

Soon after hatching, the GIT is colonized predominantly by lactic acid bacteria (LAB) including *Lactobacillus salivarius, Lactobacillus fermentum, Lactobacillus reuteri*, (Smith, 1965; Salanitro *et al.*, 1978) and members of the *Lactobacillus acidophilus* complex (Guan *et al.*, 2004). Initially the microflora is very simple, but over time becomes more complex (for an extensive review, see Sarra *et al.*, 1992). In the small intestine, the bacterial populations resemble those of adult birds within two weeks of hatching, but in the ceca the populations take from four to six weeks to fully develop (Smith 1965; Barnes, 1972). Each region of the GIT has a distinctive microflora, differing in species composition and complexity.

## The Oral Cavity

There is a lack of literature regarding the presence or absence of any commensal microbial populations in the oral cavity. It is likely that any microbes that are present are transient and move downstream into other portions of the GIT along with the ingested feed, since the feed and microbes spend very little time in the oral cavity.

## The Crop

After ingestion, the first site reached which microorganisms readily colonize is the crop. After feed enters the crop the pH of the crop drops to five due to the production of lactic acid by some members of the commensal microflora (Mead, 1997). The crop does not provide a suitable environment for strict anaerobes (bacteria which grow only in the absence of oxygen) due to the potential for exposure to oxygen and the abundance of lactobacilli. The populations of commensal bacteria colonizing the crop are extremely susceptible to changes in conditions due to dietary influences (Rubio *et al.*, 1998; Danicke *et al.*, 1999).

Initially coliforms and enterococci are present in high numbers in the crop. From hatching until four d of age, counts of lactobacilli increase and enterococci and coliform counts decrease (Fuller, 1977). Within a few days post-hatch, lactobacilli are already closely associated with the epithelial cells (Fuller and Brooker, 1974). By 14 d of age the population of the crop stabilizes with *Lactobacillus* as the predominant genus (Guan *et al.*, 2003). Until one wk of age *L. acidophilus* is the most dominant species, but by two wk of age, *L. salivarius* becomes more predominant (Guan *et al.*, 2003). The lactobacilli colonizing the crop once the microflora is fully developed are *L. johnsonii*, *L.*

*acidophilus*, *L. crispatus*, *L. gallinarium*, *L. salivarius*, and *L. reuteri* (Guan *et al.*, 2003). Small numbers of enterococci are also present (Guan *et al.*, 2003). The lactobacilli adhered to the crop form a layer two to three cells thick over the surface of the epithelium (Fuller, 1973). Micrococci, yeasts, and staphylococci are also sometimes found in the crop, as are aerotolerant anaerobes such as *C. perfringens* (Mead, 1997).

It has been established that the bacterial species present in the crop inoculate the rest of the GIT, exerting a downstream influence on the composition of the microflora throughout the GIT (Guan *et al.*, 2004). The production of lactic acid by lactobacilli in the crop, and the consequent lowering of the pH, also influence the biochemistry of downstream sections of the GIT (Fuller and Brooker, 1974).

It has been determined that the high numbers of lactobacilli in the crop are able to control populations of *E. coli* through bacteriostatic (inhibiting the growth of the bacteria) means and bacteriocidal (killing the bacteria) means (Fuller 1977). Even in chickens raised in a laboratory environment, in which only *Escherichia coli* and lactobacilli were present in the crop, the same inhibitory effect previously shown in conventional chickens was observed (Fuller, 1977).

### The Gizzard and Proventriculus

Due to the production of hydrochloric acid in the proventriculus, both the proventriculus and gizzard have a highly acidic pH ranging from one to four. This creates an environment that is very inhospitable to many microorganisms (Mead, 1997). However, there can be high levels of lactobacilli, as well as low numbers of *Escherichia coli*, enterococci, streptococci and yeasts present in the proventriculus and gizzard

(Smith, 1965). These bacterial populations are likely the result of bacteria from the crop inoculating downstream regions of the GIT (Guan *et al.*, 2004).

**The Small Intestine**

Microbial populations can be somewhat limited in the duodenum as a result of the rapid rate at which contents pass through this part of the GIT (Mead, 1997). From the time of hatch until two wk of age the duodenal microflora is comprised of a mixture of clostridia, enterococci, and enterobacteria. After two wk of age, the microflora population resembles that of an adult bird, and is comprised almost completely of *Lactobacillus* species (Smith, 1965). At this stage, the microflora in the ileum is also predominantly composed of lactobacilli, with some enterococci and enterobacteria (Smith, 1965).

Both the aerobic and anaerobic bacterial counts in the duodenum are comparable to those in the ileum, at the opposite end of the small intestine. Some research has shown that *Enterococcus*, *Staphylococcus*, and *Lactobacillus* species, along with *E. coli* are the predominant organisms in the mature small intestine (Fuller and Turvey, 1971; Salanitro *et al.,* 1978). Other studies have determined that there are only low numbers of *Escherichia coli*, enterococci, and yeasts with *Lactobacillus* as the dominant genus (Smith, 1965).

There are some obligate anaerobes present, including *Eubacterium, Propionibacterium, Clostridium*, *Gemmiger*, and *Fusobacterium* species. These anaerobes can comprise from 9 to 39% of all the bacterial strains isolated in the small intestine (Salanitro *et al.,* 1978). As of 2002, a total of 15 bacterial species had been isolated from the ileum (Gong *et al.*, 2002). More than 95% of these organisms were

Gram-positive (Gong *et al*., 2002). *Enterococcus* (*E. cecorum)* and *Lactobacillus* species (many closely related to *L. aviaries*) represented 70% of the isolates (Gong *et al*., 2002). *E. coli* has also been found in the small intestine in many instances (Salanitro *et al*., 1978; Gong *et al*., 2002).

**The Ceca and Large Intestine**

The ceca have a much more diverse and complex commensal microflora than is found anywhere else in the GIT (Mead, 1997; Gong *et al*., 2002). This is because they provide a more stable environment, and by far the most anaerobic environment of any portion of the GIT (Mead, 1997). As such, the ceca are home to many strictly anaerobic bacteria, which can be difficult to culture in laboratory settings. In many instances, attempts to culture and identify the bacterial species present in the ceca are limited by the microbiological methods used (Gong *et al*., 2002). This is evidenced by the fact that while 49 bacterial species have been found in the ceca, 25% of the isolates did not match the RNA sequence of any known bacterial species in databanks (Gong *et al*., 2002).

There is a great deal of variability in the composition of the cecal microflora between individual birds in a flock (Salanitro *et al*., 1974). Populations of each individual strain are controlled by their competition for only a few key nutritional substrates (Freter *et al*., 1983). These substrates may be endogenous, may arise from the host diet, or may be products of bacterial metabolism (Mead, 1997).

The composition of the cecal microflora undergoes dramatic changes in the first six wk of the bird's life, after which it becomes stable (Salanitro *et al*., 1974). The slow rate of cecal microflora development is a consequence of the highly sanitized barn environment and the lack of exposure to adult birds associated with commercial broiler production (Nurmi and Rantala, 1973).

There are no lactobacilli present in the ceca in the first few days post-hatch, but appear in high numbers by four d (Mead and Adams, 1975). Obligate anaerobes are predominant in the ceca after the first few days (Mead and Adams, 1975) at which point the levels of enterococci and coliforms begin to decline (Mead, 1997). Facultative anaerobes are also present at varying levels, and continue to be present throughout the life of the bird. At two wk of age the predominant genus is *Peptostreptococcus*, comprising 30% of the total bacterial population; as the bird ages, the numbers of peptostreptococci begin to decline, along with the number of lactobacilli (Barnes, 1972). Lactobacilli decrease by 100 fold by three wk of age (Barnes, 1972). By three wk of age a thick layer of bacterial cells is also present, lining the ceca. This layer, comprised of Gram-positive rod-shaped bacteria, is approximately 200 cells thick and is immediately adjacent to the cecal epithelial cells (Fuller and Turvey, 1971). After four wk, coliforms and lactobacilli are present at relatively high levels while enterococci are present at slightly lower levels. (Barnes, 1972). It is only after four wk of age that *Bifidobacterium* and *Bacteroidaceae* species are seen to be major components of the cecal microflora (Barnes, 1972).

Once mature, the cecal microflora is predominantly composed of Gram-positive anaerobic cocci, including peptostreptococci and streptococci. *Bacteroidaceae* and *Bifidobacterium* species are also present at relatively high levels, along with *Clostridium*, *Eubacterium* and *Gemmiger* species (Barnes, 1972; Barnes, 1979).

With the advent of molecular biology methods for isolating and analyzing the contents of the cecal microflora, the genuses that have been reported to be predominant in the ceca have changed. Using restriction fragment length polymorphism (RFLP) analysis, Gong *et al.*, (2002) found that the predominant groups were *Clostridium* and *Ruminococcus* species, as well as *Enterococcus cecorum*. *Bacillus*, *Eubacteria*, and *Lactobacillus* species, along with *E. coli*, have also been isolated from the ceca. However, many microbes that occur in the highest numbers in the ceca are as of yet unidentified (Gong *et al.*, 2002).

In the ceca, there is evidence of bacteria being interconnected with fibers to form a mat-like protective barrier, which is able to competitively exclude salmonella (Soerjadi *et al.*, 1982). It has also been determined that the commensal microflora can significantly reduce the pathogenicity of *C. perfringens* (Fukata, *et al.*, 1991).

## The Function of the Commensal GIT Microflora

***Competitive Exclusion.*** One of the main ways in which the commensal microflora benefits the bird is by forming a barrier that coats the lining of the GIT, thus preventing unfavorable or pathogenic microorganisms from colonizing the GIT (Isolauri *et al.,* 2001). This is referred to as competitive exclusion. Competitive exclusion also plays a role in maintaining an optimal balance between the various bacterial species of the commensal microflora. It is this bacterial antagonism that allows a few species to remain predominant in each area of the GIT while other species are present only in lower numbers (Raibaud, 1992). While competitive exclusion can successfully prevent bacterial infections, its effectiveness is often compromised by external factors. For example, when certain antibiotics are administered, they not only kill pathogenic bacteria, but also bacterial species that are a part of the commensal microflora (Raibaud, 1992).

The ability of the bird to competitively exclude pathogenic bacteria is enhanced as the bird ages and the commensal microflora becomes more complex. For example, while the microflora of an adult bird is able to competitively exclude *Campylobacter jejuni*, chicks are not capable of this until after 14 d of age (Soerjadi-Liem *et al*., 1984). However, competitive exclusion can also be accomplished early in the life of the bird by administering probiotic products to young chicks (Humbert *et al*., 1989).

Competitive exclusion not only prevents pathogens and unfavorable bacteria from attaching to the GIT mucosa, but also affects the metabolic activities of both the bird and the bacteria present in the GIT (Rowland, 1992).

***Bacterial Metabolite Production.*** In some instances, the commensal microflora can suppress bacterial metabolic processes that would result in the production of compounds toxic to the bird (Rowland, 1992). The commensal microflora may also stimulate the production of enzymes capable of rendering these toxic metabolites harmless (Rowland, 1992). Bacterial enzymes also help the bird to digest proteins, fats, and carbohydrates, as well as synthesizing certain vitamins and amino acids (Coates, 1976; Rowland, 1992). This can be valuable in situations where levels of these nutrients in the diet are insufficient to meet the nutritional needs of the bird (Rowland, 1992).

The degree to which the bird is affected by the metabolic activities of the commensal microflora depends on where the bacteria are located relative to the site of most nutrient absorption (the small intestine) (Coates, 1976). If the bacterial metabolites are formed in the cranial portion of the GIT, they are more likely to be absorbed when they reach the small intestine (Coates, 1976). This benefit of the commensal microflora is not particularly applicable to commercial broiler production, as all vitamins and nutrients are provided in levels that meet or exceed the nutritional needs of the bird.

### Costs Associated With Maintaining the GIT Microflora

While there are benefits to having a healthy and well developed GIT microflora, there are also costs to the bird. Some bacteria in the GIT are able to dehydroxylate, deconjugate, and dehydrogenate bile acids, resulting in a lower availability of digestible fats to the bird (Knarreborg *et al.*, 2002). Some bacterial species in the GIT are also responsible for the deconjugation and reduction of billirubin, resulting in an increased energy and nutrient cost to the bird, since more must be synthesized *de novo* (Tannock, 1998). The species in the GIT microflora produce intestinal gas, increase oxygen consumption and heart rate, cause thickening of the intestinal wall, increase the intestinal surface area, cause faster enterocyte replacement, increase peristaltic movement, raise body temperature, and cause larger lymph nodes (for an extensive review, see Tannock, 2001). All of these functions of the GIT microflora contribute to an increased amount of energy required by the bird for maintenance.

In addition, the commensal microflora, in attempting to meet its own nutritional needs, may compete with the bird for nutrients in the GIT (Coates, 1976). For example, some bacteria are able to decarboxylate or deaminate amino acids (Rowland, 1992). While this action is beneficial to the bacterium, and helps to meet its own nutritional needs, it has detrimental consequences for the bird, rendering the amino acids unavailable (Rowland, 1992).

## Factors Affecting The GIT Microflora

***Ability to Adhere.*** Adherence of commensal microflora species is important because this is the first step in infection for a pathogenic microorganism if it were to enter the GIT. As such, one of the main determinants of prevalence is the ability of the bacterium to adhere to the epithelial cells lining the GIT (Fuller, 1973; Fuller, 1977; Stavric *et al.*, 1991).

This ability provides an ecological advantage, allowing the commensal microflora to competitively exclude other organisms from attaching to the epithelium, and in turn enables the maintenance of stable population levels (Fuller 1973; Fuller, 1977). Attachment of the microflora to the wall of the GIT is a good way to evade the dangers of living in an environment such as the GIT that is constantly in motion, but is by no means a simple procedure. Attachment occurs through a series of steps, and can be influenced by many factors (Jones *et al.*, 1980).

Adhesion of *Enterococcus faecium* in the duodenum has also been shown in chick epithelial cells (Fuller *et al.*, 1981). Together, enterococci, lactobacilli and several other species are responsible for the competitive exclusion of pathogens in the small intestine (Fuller *et al.*, 1981). However, the attachment of lactobacilli in the crop, and the ability to competitively exclude pathogens is more thoroughly researched. This may be due to the aerotolerant nature of microorganisms in the crop, making them easier to culture and work within a laboratory setting. In most cases, bacterial strains originally isolated from the GIT of the chicken are most able to adhere to the epithelial cells of the chicken crop (Fuller, 1973).

***Pathogenic Bacteria and Disease*.** The transmission of pathogens, or other bacteria not normally present in the GIT, to young chicks can delay or altogether obstruct the development of a healthy commensal microflora. For example, when *Salmonella*-contaminated eggs were artificially introduced into an incubator, the GIT of more than half of the chicks were infected with *Salmonella* at hatching (Cason *et al*., 1994). Whereas in commercial situations *Salmonella* contamination of incubators and hatchers is rare (Cason *et al*., 1994), the artificial introduction of *Salmonella* in the above study showcases the extreme vulnerability of young birds to bacterial infections. Once bacterial species such as *Salmonella* colonize the GIT, the restoration of a healthy commensal microflora is very difficult (Watkins *et al*., 1982; Cason *et al*., 1994; Jin *et al*., 1996).

***Effects of Stress on GIT Bacterial Populations.*** Bacterial populations in the GIT are subject to many different environmental, developmental, and production stressors (Hume *et al*., 2003). These stressors combined with a pre-existing disease state can cause the numbers of microbes in the GIT to fluctuate drastically (Barnes, 1979). For example, if some event occurs and lactobacilli populations in the crop become limited, *Streptococcus* species and *Enterobacteriaceae* will quickly emerge as predominant (Fuller, 1977). Post-infection it is difficult to reinstate normal numbers of lactobacilli even when the bird is orally gavaged with high doses of lactobacilli (Jin *et al*., 1996).

Exposure to environmental, nutritional, and disease related stressors also increases the risk that pathogenic bacteria will colonize the GIT wall (Sarra *et al*., 1992), resulting in an imbalance of the normal microflora, and reducing the immune response (Jin *et al*., 1998). There are several ways in which these pathogenic or non-pathogenic microorganisms can cause growth depression in poultry. These include toxin production, utilization of nutrients that are essential to the bird, and the suppression of other microorganisms that synthesize vitamins or growth factors (Mead, 1997). This may make probiotics an effective tool in helping to maintain a healthy microflora and prevent growth depression during times of stress (Fairchild *et al.*, 2001).

## Antibiotic Use in Broiler Production

Antibiotics have long been used by North American poultry producers to achieve poultry production goals. Antibiotics are used not only as treatment and prophylaxis (prevention) for many diseases caused by pathogenic bacteria, but are also routinely administered at sub-therapeutic levels for growth promotion purposes. The addition of sub-therapeutic levels of antibiotics in commercial poultry diets became a mainstream practice in the late 1950's, and has remained a primary method of preventing growth depression due to bacterial infection (Yeo and Kim, 1997). The use of antibiotics in this manner protects birds from colonization of the GIT by pathogenic bacteria, resulting in improved performance and health (Eyssen and DeSomer, 1967; Engberg *et al.*, 2000).

Although antibiotics provide birds with health benefits, antibiotics accomplish these goals in commercial poultry by killing some of the bacterial species present in the GIT; this includes both pathogenic bacteria and those which are an important part of the commensal microflora, such as *L. salivarius* (Sheldon and Essary, 1982; Engberg *et al.*, 2000). Antibiotic administration also results in morphological changes in the GIT, including thinning of the intestinal wall, as is seen in birds with no GIT microflora (Sheldon and Essary, 1982). There are also consumer concern issues involved with administering antibiotics to poultry.

***Antibiotic Resistance by Pathogenic Bacteria***. Consumers have become increasingly concerned with the perceived risks associated with the consumption of meat products that possibly contain antibiotic residues (Jin *et al.*, 1998). In addition, some antibiotics are losing their effectiveness in treating bacterial infections due to an increase in antibiotic resistance by both human and poultry pathogens (Fairchild *et al.*, 1998). Antibiotic use at low levels in animal feeds contributes to the development of antibiotic-resistant microorganisms. This resistance may be passed on to microorganisms with the capacity to infect humans, resulting in infections caused by bacteria which are drug-resistant, and therefore difficult to treat (Howe *et al.*, 1976). Antibiotic resistance can be either intrinsic or acquired. Intrinsic resistance is a characteristic of the species or genus, and is not easily transmissible to other organisms. Acquired resistance, on the other hand, is the result of a genetic mutation or having foreign DNA from another microorganism incorporated into pathogenic bacterial DNA (Mattila-Sandholm *et al.*, 1999). One example of how acquired resistance can occur is when producers choose to use antibiotics prophylactically to prevent cellulitis, caused by *E. coli*. However, because *E. coli* exhibits high heterogenicity (differences in genotype occurring as the result of reproduction), the possibility of creating one or more strains resistant to antibiotics is great (Estrada *et al.*, 2001). In fact, *E. coli* is one bacterial species that is of great concern with respect to antibiotic resistance (Fairchild *et al.*, 2001). The fear of antibiotic resistance, both in the scientific community and by consumers, mandates that alternatives to antibiotic supplementation for improving growth be found (Estrada *et al.*, 2001).

The production of microbiologically safe poultry products is a complex process due to the many stages in poultry production and processing during which the birds or carcasses can become contaminated with bacterial pathogens (Blankenship *et al.*, 1993). It is for this reason that antibiotics are commonly used to prevent, as well as treat, bacterial infections. Manipulation of the commensal microflora using alternative measures such as probiotics may be able to accomplish this same goal without causing antibiotic resistance.

***Antibiotic Resistance by Commensal Bacteria***. There is research confirming that beneficial bacterial species are sometimes resistant to antibiotics. Resistance to bacitracin in *L. acidophilus* strains exists, but is not widespread (Dutta and Devriese, 1981) whereas macrolide and lincosamide resistant lactic acid bacteria (LAB) are common (Dutta and Devriese, 1984). It is unclear whether this is the result of acquired antibiotic resistance, or if it is an intrinsic trait of these bacterial species. Many LAB are intrinsically resistant to vancomycin. However, these species have been used as probiotics in humans for quite some time with no incidence of this resistance being transferred to other microorganisms (Mattila-Sandholm *et al.*, 1999). From the available literature, it does not appear that the scientific community anticipates any problems with pathogenic bacterial strains acquiring these antibiotic resistant genes from probiotic bacteria. This indicates that probiotics may be able to work either alone, or in conjunction with therapeutic antibiotics when disease outbreaks do occur to re-establish a healthy microflora. In a study by Seuna and Nurmi (1979), broilers were experimentally infected with *Salmonella*, and no antimicrobial regime alone could reduce the incidence of reoccurrence of infection. However, when birds were given antibiotics and treated with a culture containing the cecal microflora from an adult bird, the number of chicks that became reinfected was reduced (Seuna and Nurmi, 1979).

Some European countries, such as Denmark, have already banned the use of all antibiotics as feed additives at sub-therapeutic levels, while others, such as the United Kingdom, have imposed very strict guidelines on the usage of some antibiotics while banning others altogether. There appears to be a worldwide trend toward decreasing, and eventually eliminating the use of sub-therapeutic levels of antibiotics in animal agriculture (Jin *et al.*, 1998).

The use of probiotic products is one method that is becoming popular to partially control the colonization of the gut by pathogenic bacterial species such as *Salmonella* (Blankenship *et al.*, 1993). Thus, if effective probiotics are developed commercially, they may provide a safe and effective alternative to the use of constant low doses of antibiotics in poultry production.

## PROBIOTICS IN POULTRY PRODUCTION

Although the concept of probiotics as poultry feed supplements only goes back to the mid 1970's, the idea of using live microbial supplements to improve human health and prevent food spoilage is thousands of years old (for a review see Fuller, 1992). The definition of probiotics has changed a great deal over the past 40 years, and it is only over the last 20 years or so that there has been any kind of consensus in the scientific community on the proper definition of the word (Fuller, 1992). Lilly & Stillwell (1965) used the term to describe substances that were secreted by a microorganism that stimulated the growth of another microorganism. Later, Sperti, (1971) used the term to define tissue extracts that were able to stimulate microbial growth. In an attempt to better refine the definition and improve its accuracy, Fuller (1989) defined probiotics as "a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance". This more accepted definition draws specific attention to the fact that a probiotic must be viable, thus excluding antibiotics, organic acids, and any number of substances which would have been included under past definitions. The term probiotic is now solely used to refer to live microbial cultures that are isolated from the GIT of humans or animals

It is inevitable that a microbial population will develop in the GIT soon after hatching. The bacterial strains that are the first to become established are often the predictors of which microorganisms will persist in the GIT throughout the bird's life (Jernigan *et al.*, 1985). In the past, in traditional production systems, the chicks were hatched in the presence of the hen, and were therefore exposed to the feces of the adult shortly after hatching by pecking the ground and other objects contaminated with feces in the rearing environment. Consumption of adult feces by the newly hatched chick inoculated the chick's GIT with a healthy adult microflora, thereby protecting the chick against the colonization of the GIT by undesirable bacteria and pathogens. The use of probiotics in modern production systems attempts to provide the same benefit as consumption of adult feces by intentionally introducing beneficial bacteria to the chicks shortly after hatching.

Probiotics work on the principle that if the development of the commensal microflora in the GIT of the bird can be accelerated, a GIT microflora composed of beneficial bacterial species can be established. This would result in the competitive exclusion of pathogens and transient microorganisms early in the life of the bird, before the commensal microflora would otherwise be sufficiently developed to perform this function (Humbert *et al.,* 1989). Another goal of administering probiotics is to maintain a balance of the appropriate numbers of each beneficial bacterial species in the GIT of all birds in the flock throughout production (Haddadin *et al.*, 1996). This strikes a balance between the benefits offered by competitive exclusion and the competition of the probiotic bacteria with the bird for essential nutrients in the GIT (Jernigan *et al.*, 1985). However, without the use of probiotics, or other means to manipulate the microflora composition, this balance can be very difficult to achieve under commercial conditions (Jernigan *et al.*, 1985). Supplementation with probiotics could boost the numbers of beneficial bacteria in the GIT, making the balance easier to maintain (Jernigan *et al.*, 1985).

For the purpose of the following discussion, probiotics can be divided into two main categories. Undefined probiotics are those that are composed of a bacterial culture obtained from the fecal or cecal matter of adult birds. The bacterial composition of undefined cultures is not precisely known. In contrast, defined probiotics are those in which the exact bacterial composition of the culture is known. Defined probiotics can be either simple (containing one or a few bacterial strains) or complex (containing a mixture of as many as 30 or more bacterial strains).

### Potential Benefits of Probiotic Supplementation in Broilers

***Production Efficiency.*** The administration of either undefined, or complex defined cultures of probiotic bacteria to poultry has been shown to increase weight gains compared to broilers not given probiotics (Nurmi and Rantala, 1973; Tortuero, 1973).

Enhanced mucosal immunity (Dalloul *et al.*, 2003), decreased colonization of the GIT by unfavorable bacteria (Chambers and Lu, 2002), and an improved metabolizable energy (ME) value of feed (Schneitz *et al.*, 1998) have also been noted in probiotic treated broilers compared to broilers not treated with probiotics. In contrast, another study using complex, defined products showed no differences in FCR, body weights, or mortality between treated and untreated broilers (Palmu and Camelin, 1997). Other research determined that there were no adverse effects on chick performance noted due to the application of a complex defined probiotic as a spray (Blankenship *et al.*, 1993) or in the drinking water (Wierup *et al.*, 1988). However, no production benefits were observed in the above studies.

Research examining simple defined probiotics in broilers has had mixed results. Some studies have shown no improvements in FCR (Watkins and Kratzer, 1983; Watkins and Kratzer, 1984; Estrada *et al.*, 2001; Huang *et al.*, 2004), body weights (Watkins and Kratzer, 1983; Watkins and Kratzer, 1984; Bilgili and Moran, 1990; Estrada *et al.*, 2001; Hofacre *et al.*, 2003), or mortality (Watkins and Kratzer, 1983; Watkins and Kratzer, 1984; Bilgili and Moran, 1990; Jin *et al.*, 1998; Zulkifli *et al.*, 2000; Estrada *et al.*, 2001) over broilers not given probiotics. Contrasting results, in which simple defined products were administered, have shown increased broiler body weights and weight gains (Mohan *et al.*, 1996; Yeo and Kim, 1997; Jin *et al.*, 1998; Zulkifli *et al.*, 2000), improved FCR (Jin *et al.*, 1998; Zulkifli *et al.*, 2000) and lower serum cholesterol levels (Mohan *et al.*, 1996). In some cases, initial improvements in the performance of poultry have been reported, but there were no differences between the birds given probiotics and the control group by the time the birds reached market age. Such was the case when *Bacillus subtilis*

(Jiraphocakul *et al.*, 1990) and a simple combination of *Lactobacillus* strains (Potter *et al.*, 1979) were administered to turkeys.

***Increased GIT Enzyme Activity.*** Jin *et al.*, (2000) measured digestive enzyme activity as a means of measuring the effect of LAB on digestion in broilers. They found that including either *L. acidophilus* or a mixture of *Lactobacillus* strains as a feed additive resulted in increased levels of amylase activity in the small intestine. This would improve the efficiency of digestion of carbohydrates by the broiler. Feeding these same *Lactobacillus* strains also decreased levels of both intestinal and fecal $\beta$-glucosidase, which produces toxic and carcinogenic substances. Lower $\beta$-glucosidase levels would prevent the hydrolysis of glucosides to form compounds, such as cyanide, that are toxic to the broiler (Jin *et al.*, 2000).

### Impact on Food Safety

Since food safety is rapidly emerging as a major consideration for consumers, anything that can reduce the incidence of poultry meat contaminated with *Salmonella* and other pathogens would benefit both consumers and the poultry industry. Since the susceptibility of chickens to *Salmonella* colonization is influenced by bird health (Kubena *et al.*, 2001), probiotics may aid in improving food safety (Snoeyenbos *et al.*, 1978; Barnes *et al.*, 1980; Pivnick *et al.*, 1981; Stavric *et al.*, 1985; Gleeson *et al.*, 1989; Chambers and Lu, 2002). By reducing the number of *Salmonella*-infected birds arriving at the processing plant, the incidence of cross-contamination of carcasses and the contamination of meat from these carcasses could be reduced (Palmu and Camelin, 1997).

### Reduction in Colonization of the GIT by Pathogens

In their landmark 1973 study, Nurmi and Rantala supplemented the diet of chicks with cecal matter, containing an undefined cecal microflora from healthy adult birds. This resulted in decreased colonization of the GIT by *Salmonella*. This was the first of many studies conducted to investigate the effects of a variety of different beneficial bacterial strains, and combinations of strains, on poultry production efficiency and food safety.

Blankenship *et al.*, (1993) investigated the effect of administering an undefined probiotic culture to broilers on *Salmonella* contamination of carcasses at processing. The culture was applied in two doses, as a spray in the hatcher, and in the first drinking water after the chicks were placed. When chicks were subsequently challenged with S. Typhimurium, the S. Typhimurium did not as readily colonize the broilers treated with probiotics compared to the untreated broilers. This was evidenced by the fact that the carcasses of 41% of the untreated birds were *Salmonella* positive compared to only 10% of carcasses from the probiotic treated group (Blankenship *et al.*, 1993). This highlights the fact that probiotics are not a 'magic bullet' to solve all food safety issues, but may be useful as part of an integrated approach in managing broilers to reduce the levels of pathogenic bacteria present during production and at processing. Similar results were found in a study using a complex, defined culture applied as a spray at hatching (Chambers and Lu, 2002).

In another study, cecal volatile fatty acid levels were used as an indicator of the ability of a complex defined probiotic to colonize the ceca of chicks. It was determined that chicks sprayed with the probiotic at hatch had significantly higher cecal volatile fatty acid concentrations than untreated chicks (Kubena *et al.*, 2001). This indicated that the probiotic bacterial strains were able to colonize the GIT. Once the probiotic treatment had been administered, the chicks were orally challenged with *Salmonella*. After the challenge, only 10% of broiler flocks treated with probiotics had ceca colonized by *Salmonella*, compared to 85% of untreated broiler flocks (Kubena *et al*., 2001).

In a field study encompassing more than 1,200,000 broilers, Palmu and Camelin (1997) examined the effect of a complex defined commercial probiotic (applied as a spray at hatch) on the incidence of *Salmonella* contamination in broilers. During production, only 6% of litter samples from the flocks treated with probiotics tested positive for *Salmonella*, compared to 42% of litter samples from control flocks. The same trend was evident at the processing plant, with fewer flocks treated with probiotics testing positive for *Salmonella* than control flocks (Palmu and Camelin, 1997).

Research has also been conducted to assess the efficacy of single probiotic strains in reducing colonization of the GIT by unfavorable bacteria. Both *L. acidophilus* and *L. fermentum* were able to reduce the degree to which some *Salmonella* strains colonized the chick GIT, but neither was effective in competitively excluding S. Enteritidis (Jin *et al*., 1996). However, *L. salivarius* was shown to provide transient protection against colonization of the GIT by S. Enteritidis, for as long as the probiotic was administered (Pascual *et al*., 1999). Silva *et al*., (1981) administered an undefined adult cecal microflora to chicks and determined that this treatment partially protected the GIT from colonization by *Salmonella gallinarium*, the bacterium that causes fowl typhoid (Silva *et al*., 1981). In some instances it has also been demonstrated that one undesirable organism may competitively exclude another. For example, *E. coli* is able to prevent *Salmonella* from colonizing the chick GIT (Baba *et al*., 1991). In another study, *B. subtilis* effectively prevented colonization of the GIT by *E. coli* for up to 35 days following a single dose of the probiotic culture (LaRagione *et al.*, 2001).

Further research showed that one d old chicks treated with undefined cecal microflora had a reduction in *Salmonella* colonization of the GIT. There was an increase in the levels of cecal propionic acid in the birds given the culture, indicating increased protection against *Salmonella* colonization (Nisbet *et al.*, 1993).

### Proposed Modes of Action of Probiotics

As mentioned previously, the immature development of the GIT microflora of chicks makes them very susceptible to colonization by pathogenic bacteria species (Kubena *et al.*, 2001). It is thought that probiotics prevent infections of pathogenic or undesirable bacterial species by establishing a bacterial population in the GIT before pathogenic bacteria have the opportunity to colonize the GIT. This results in both the formation of a "protective barrier bacterial population" (Fairchild *et al.*, 2001) in the GIT, as well as stimulation of the development of the commensal microflora at a very young age. Rather than waiting for the microflora to develop naturally, this early establishment shortly after hatching is important. Once *Salmonella* gains access and begins to colonize the GIT, even the reintroduction of bacterial species from the commensal microflora is not effective in displacing *Salmonella* from GIT epithelial cells (Cason *et al.*, 1994; Jin *et al.*, 1996).

The exact mode(s) of action for how probiotics achieve beneficial production and health effects *in vivo* are unclear, however, several mechanisms have been proposed over the past few decades.

***Competitive Exclusion of Pathogens.*** Competitive exclusion was first explained by Nurmi and Rantala (1973). Effective probiotic bacterial strains, or combinations of strains competitively exclude pathogens and unfavorable organisms in the same way as the commensal microflora. The only difference is that probiotics enable this function at a younger age, before the commensal microflora are sufficiently developed to accomplish this function on their own (Humbert *et al.*, 1989). The vulnerability of newly hatched chicks to pathogens is demonstrated by the fact that chicks can be infected by only a single cell of *Salmonella* (Bailey *et al.,* 1988). Older birds, on the other hand, possess some immunity to these bacteria because of the commensal GIT microflora. Hence, the introduction of GIT microflora from an adult bird to newly hatched chicks can enhance the chicks' resistance to colonization of the GIT by *Salmonella* (Bailey *et al.*, 1988).

When probiotic bacteria are administered in high numbers, it is thought that the beneficial bacteria take up all the binding sites in the GIT (Nisbet *et al.*, 1993). This leaves pathogenic bacterial species unable to attach to the epithelium of the GIT (Nurmi and Rantala, 1973; Snoeyenbos *et al.*, 1978; Nisbet *et al.*, 1993; Yeo and Kim, 1997), and thus the pathogenic bacteria are flushed out with the feces. The most studied example of competitive exclusion is the colonization of the crop walls by lactobacilli, which compete for both physical space, as well as nutrients with pathogens and unfavorable bacteria such as *E. coli* (Fuller, 1977; Watkins and Kratzer, 1983).

***Lactic Acid Production.*** The second proposed mode of action for probiotics is lactic acid production. Lactobacilli are a major component of the chicken GIT microflora, and as such are commonly included in probiotic products. Lactobacilli produce lactic acid as an end product of bacterial metabolism. This lactic acid creates a low pH environment, which has an inhibitory effect on the growth and survival of acid sensitive pathogenic bacterial species (Fuller, 1977; Chateau *et al.,* 1993).

***Alteration of Bacterial Metabolism.*** There are also other ways in which probiotics influence the metabolism of the bacteria present in the GIT. In the case of probiotic strains, the bacteria benefit from the favorable environment and flow of nutrients in the GIT (Jernigan *et al.,* 1985). The bird benefits from maintaining beneficial bacterial species that do not contribute to a disease state and may even improve the health of the bird (Jernigan *et al.*, 1985).

The metabolic activities of both bird and bacteria are influenced by the level of activity of different digestive enzymes. It has been shown that *Lactobacillus* strains isolated from chicken intestine possess amylolytic activity (Jin *et al.*, 2000). Bacterial metabolism can also be influenced by changes in the metabolic activity of other bacteria (Chiang and Hsieh, 1995). For example, ammonia production by bacteria may influence bacterial metabolism. Chiang and Hsieh (1995) found lower ammonia concentrations in the feces of broiler chickens fed probiotics. Suppression of urease activity in poultry fed probiotics has also been reported (Yeo and Kim, 1997). Urease is produced by many bacterial species that are responsible for growth depression in poultry, and decreased urease levels could indicate a reduction in the numbers of these bacteria present in the GIT (Yeo and Kim, 1997). It has also been suggested that probiotics influence the metabolic activities of pathogenic bacteria through the production of inhibitory substances, such as bacteriocins (Yeo and Kim, 1997).

***Production of Volatile Fatty Acids.*** Another proposed mechanism is the production of short chain volatile fatty acids, namely acetic, propionic, and butyric acids, by probiotic bacteria (Barnes, 1972; Barnes, 1979). One study showed a 22-fold increase in the levels of cecal propionic acid in broilers given an undefined probiotic compared to broilers given no probiotic (Nisbet *et al.*, 1993). These volatile fatty acids have the capacity to inhibit *Salmonella* colonization both *in vitro*, and in broilers *in vivo* (Barnes, 1972).

***Stimulation of Immune Function.*** Probiotics may promote endogenous host defense mechanisms (Perdigon *et al.*, 1995; Isolauri *et al.*, 2001), and may also improve humoral immune responses, and stimulate nonspecific host resistance to microbial pathogens, thus facilitating the exclusion of these pathogens from the GIT (Isolauri *et al.*, 2001). In broilers it has been shown that broilers treated with probiotics had higher antibody levels than control birds after a challenge dose of *Eimeria acervulina* was administered (Dalloul *et al.*, 2003)

## Factors Affecting the Efficacy of Probiotics

One reason that probiotics are not widely used in commercial poultry production is the conflicting reports on their efficacy. This is likely the result of inconsistencies between studies with regard to the bacterial strains or mixtures of strains administered, and the methods in which they were prepared and administered. These discrepancies could also reflect differences in the age of the birds at the time of administration, sanitation of the environment in which they were reared, or the dose of probiotic bacteria administered. However, whereas the available research differs drastically in the experimental designs used and the results obtained, the resulting body of literature does allow general criteria for an effective probiotic to be described.

***General Factors Required for an Effective Probiotic.*** In order to be effective in broilers, probiotic products should contain bacterial species normally found in the GIT of a healthy adult chicken. Those bacterial species should be defined according to a current, valid taxonomic system and should remain viable for the entire shelf life of the product in the numbers specified by the manufacturer. These bacteria must also be able to survive transit through the GIT to the intended site of colonization and should have the ability to induce some beneficial effect in the GIT while not impairing the health of the host (Reuter *et al.*, 2002). When administering probiotics, it is important to ensure that the method of administration is one that will allow the probiotic to survive passage so it will be alive and intact in order to attach to epithelial cells (Jin *et al.*, 1998). For example, if a strictly anaerobic probiotic strain of bacteria is used, it may not be suitable for mixing in feed where it will be exposed to oxygen for quite some time before being ingested by the bird.

***Host Specificity.*** Probiotic products should be host-specific since it has been shown that products composed of bacterial strains isolated from the GIT of an adult chicken are more effective in chickens than those composed of bacterial strains isolated from other animals (Cox *et al.*, 2001). There are some instances where bacterial strains derived from chickens have been effective in preventing colonization of the GIT by pathogens in turkeys and vice versa (Weinack *et al.*, 1982; Hollister *et al.*, 1999). However, for the most part, bacteria will not attach to the GIT epithelial cells in animal species from which they have not been isolated (Jiraphocakul *et al.*, 1990; Haddadin *et al.*, 1996). This is referred to as host specificity.

***Factors to Consider for Inclusion of Probiotics in Feed or Water.*** There are certain criteria that must be met in order for bacterial strains to be suitable for addition to the diet. The chosen bacterial strain must remain viable in the feed during the period between feed mixing, and consumption by the bird. The organism must also be resistant to bile salts, stomach acids, and all other rigors of the GIT (Haddadin *et al.*, 1996).

When undertaking *in vivo* studies, especially on a commercial scale, there are additional factors that must be taken into account. For example, the effects of other feed additives must be considered to ensure that they will not negatively impact the efficacy of the probiotics. Bailey *et al.* (1988) investigated the effects of various anticoccidial and antimicrobial feed additives on probiotic cultures. It was determined that inclusion of nicarbazin (an anticoccidial) and bacitracin (an antimicrobial) decreased the protection offered by the addition of an undefined probiotic culture (Bailey *et al.,* 1988). In this study, other feed additives were tested, and had no effect on the protection offered by the probiotic. This was confirmed by the lack of *Salmonella* colonization of the GIT after the birds were orally challenged with *Salmonella* (Bailey *et al.*, 1988). From this, it can be inferred that the effect of certain feed additives on probiotics depends on the characteristics of the particular bacterial strains used.

When the effectiveness of a probiotic product such as Interbac®[1] or PREEMPT®[2] depends on ingestion of a liquid sprayed on the birds, factors which may influence preening activity can alter colonization of the GIT by probiotic bacterial species (Caldwell *et al.*, 2001). It has been shown that the addition of any color to an uncolored liquid before spraying provided an increase in preening behavior. The use of darker colors resulted in more preening compared to light colors (Caldwell *et al.*, 2001).

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[1] Intervet Canada, Inc., 250 Water Street, Whitby, ON, Canada, L1N 9T5.
[2] MS Bioscience, PO Box 278, Illinois and Water Street, Dundee, IL, 60118.

There is also the potential for different feed ingredients to influence the activity of probiotics, or the bacteria that they are designed to competitively exclude. For example, diets high in wheat or barley increase the viscosity of the digesta in the intestine, creating a more anaerobic environment; this is favorable for pathogens such as *C. perfringens* (Choct *et al.*, 1996). Wheat and barley are among the feed ingredients that are widely known to have this effect on digesta viscosity, resulting in an increased incidence of NE (Riddell and Kong, 1992; Annett *et al.*, 2002).

***Microbiological Composition of Probiotic Products.*** For the desired beneficial effects to result, the right species must be used in a probiotic product. A thorough understanding of the bacterial species populating the GIT is required before effective probiotic products can be developed (Tannock, 2001; Gong *et al.*, 2002). Much of this basic information on species composition is still lacking. For this reason, numerous species have been included in many different combinations in probiotic studies; the differences in the bacterial composition and complexity of the probiotic preparations used may contribute to the conflicting results with regard to the efficacy of the cultures used.

What can be gleaned from the available literature is that whereas single strains or combinations of a few strains are generally effective when orally gavaged to small numbers of birds under laboratory conditions, these same strains have reduced effectiveness when applied in a setting similar to commercial production (Baba *et al.*, 1991; LaRagione *et al.*, 2001). By far the most effective probiotic products seem to be those that are composed of many bacterial strains or of undefined cecal contents collected from an adult bird of the same species (Gleeson *et al.,* 1989; Baba *et al.,* 1991; Fukata *et al*., 1991; Nisbet *et al*., 1993, 1998; Hofacre *et al*., 2003). Bacterial composition is also important since different components of the commensal microflora are responsible for competitively excluding different pathogens or undesirable microorganisms. This is evidenced by the fact that the same bacterial species are generally not able to exclude both *Salmonella* and *Campylobacter* species (for a review see Mead, 2002).

If probiotics and antibiotics are to be used together, the choice of probiotic strains is crucial to the success of this combined approach (McReynolds *et al*., 2000). Some of the antibiotics that have traditionally been used prophylactically to prevent disease and improve growth in poultry can kill not only the target pathogen, but some of the bacteria present in the commensal GIT microflora as well (Chambers and Lu, 2002). This actually makes the birds more susceptible to pathogenic bacteria and inhibits what natural protection the bird has from bacterial infections (McReynolds *et al*., 2000; Chambers and Lu, 2002). This effectively makes the bird reliant on constant doses of sub-therapeutic antibiotics to prevent colonization of the GIT by opportunistic pathogens (Fukata *et al*., 1991).

### Regulatory Aspects

In Canada, the Veterinary Biologics Section (VBS) of the Animal Health and Production Division of the Canadian Food Inspection Agency (CFIA) is responsible for licensing and regulating the use of veterinary biologics, including probiotics (CFIA, 2001). The VBS uses the term "direct-fed viable microbial product" as opposed to probiotic. In order for a product to become licensed it is tested according to four criteria:

purity, potency, safety, and efficacy (CFIA, 2001). When applying to have a probiotic product registered and licensed, the manufacturer must provide proof of efficacy in the form of data from three separate trials, each containing an appropriate control group. In the case of broiler trials, both treated and control birds must receive an approved coccidiostat as well. Strains of bacteria must be clearly identified in each product, and a report indicating the experimental procedures used in identifying and quantifying the bacteria must also be submitted to the VBS prior to licensing (CFIA, 1997).

At present the CFIA has only approved two probiotic products for use in avian species (including chickens, turkeys, ducks and geese) in Canada. The first product is Interbac®, which can be administered either as a spray at the time of hatch or in the drinking water. For broilers, if it is administered in the water it should be at placement and again at approximately three wk of age. The second product is Pro-Avi®, a powdered feed additive to be administered throughout the production period. The research contained within this thesis was conducted to evaluate the effectiveness of the probiotics currently available for use in poultry in Canada.

# REFERENCES

Alberta Chicken Producers, 2004a. Alberta production by cornish broiler roaster. http://www.chicken.ab.ca/statistics_ab_production.html. Accessed July 10, 2004.

Alberta Chicken Producers, 2004b. FAQ's.http://www.chicken.ab.ca/diduknow_faq.html. Accessed July 10, 2004.

Al-Sheikhly, F., and R. B. Truscott, 1977. The interaction of *Clostridium perfringens* and its toxins in the production of necrotic enteritis of chickens. Avian Dis. 21:256-263.

Annett, C. B., J. R. Viste, M. Chirino-Trejo, H. L. Classen, D. M. Middelton, and E. Simko, 2002. Necrotic enteritis: effect of barley, wheat and corn diets on proliferation of *Clostridium perfringens* type A. Avian Pathol. 31:599-602.

Aviagen Inc., 2004. North American Information. http://www.ross-na.aviagen.com/. Accessed July 12, 2004.

Baba, E., S. Nagaishi, T. Fukata, and A. Arakawa. 1991. The role of intestinal microflora on the prevention of Salmonella colonization in gnotobiotic chickens. Poult. Sci. 70:1902-1907.

Bailey, J. S., L. C. Blankenship, N. J. Stern, N. A. Cox, and F. McHan, 1988. Effect of anticoccidial and antimicrobial feed additives on prevention of *Salmonella* colonization of chicks treated with anaerobic cultures of chicken feces. Avian Dis. 32:324-329.

Barnes, E. M., 1972. The avian intestinal flora with particular reference to the possible ecological significance of the cecal anaerobic bacteria. Am. J. Clin. Nutr. 25:1475-1479.

Barnes, E. M., 1979. The intestinal microflora of poultry and game birds during life and after storage. J. Appl. Bacteriol. 46:407-419.

Barnes, E. M., C. S. Impey, and D. M. Cooper, 1980. Manipulation of the crop and intertinal flora of the newly hatched chick. Am. J. Clin. Nutr. 33:2426-2433.

Barrow, P. A., 1992. Probiotics for chickens. Pages 255-257 in Probiotics: The Scientific Basis. R. Fuller (ed.). Chapman and Hall, London, UK.

Bilgili, S. F., and E. T. Moran Jr., 1990. Influence of whey and probiotic-supplemented withdrawal feed on the retention of *Salmonella* intubated into market age broilers. Poult. Sci. 69:1670-1674.

Blankenship, L. C., J. S. Bailey, N. A. Cox, N. J. Stern, R. Brewer, and O. Williams, 1993. Two-step mucosal competitive exclusion flora treatment to diminish salmonellae in commercial broiler chickens. Poult. Sci. 72:1667-1672.

Bray, D. F., and E. L. Iton, 1962. The effect of egg weight on strain differences in embryonic and post-embryonic growth in the domestic fowl. Br. Poult. Sci. 3:175-188.

Caldwell, D.Y., S. D. Young, D. J. Caldwell, R. W. Moore, and B. M. Hargis, 2001. Interaction of color and photointensity on preening behavior and ingestion of spray-applied biologics. J. Appl. Poult. Res. 10:112-116.

Cason, J. A., N. A. Cox, and J. S. Bailey, 1994. Transmission of *Salmonella typhimurium* during hatching of broiler chicks. Avian Dis. 38:583-588.

CFIA, 1997. T-3-143 Regulation of viable microbial products for oral administration to livestock.http://www.inspection.gc.ca/english/anima/feebet/tradememt/t3143e.shtml. Accessed June 23, 2004

CFIA, 2001. Veterinary biologics section. http://www.inspection.gc.ca/english/anima/vetbio/vbpbve.shtml. Accessed June 23, 2004.

Chambers, J. R., and X. Lu. 2002. Probiotics and maternal vaccination for *Salmonella* control in broiler chickens. J. Appl. Poult. Res. 11:320-327.

Chateau, N., I. Castellanos, and A. M. Deschamps, 1993. Distribution of pathogen inhibition in the *Lactobacillus* isolates of a commercial probiotic consortium. J. Appl. Bacteriol. 74: 36-40.

Chiang, S. H., and W. M. Hsieh, 1995. Effect of direct-fed microorganisms on broiler growth performance and litter ammonia level. Asian-Australian J. Anim. Sci. 8:159-162.

Chicken Farmers of Canada, 2003. Chicken data handbook. http://www.chicken.ca/pdfs/Data_Handbook_2003.pdf. Accessed July 12, 2004.

Choct, M., R. J. Hughes, J. Wang, M. R. Bedford, A. J. Morgan, and G. Annison, 1996. Increased small intestinal fermentation is partly responsible for the anti-nutritive activity of non-starch-polysaccharides in chickens. Br. Poult. Sci. 37:609-621.

Clauss, W., V. Dantzer, and E. Skadhauge, 1991. Aldosterone modulates electrogenic Cl secretion in the colon of the hen (Gallus domesticus). Am. J. Physiol. 261:R1533-R1541.

Coates, M. E., 1976. Influence of the gut microflora on digestion and absorption. Pages 179-191 in Digestion in the Fowl. K. N. Boorman and B. M. Freeman (ed.). British Poultry Science Ltd., Edinburgh, UK.

Coates, M. E., and R. Fuller, 1977. The gnotobiotic animal in the study of gut microbiology. Pages 311-346 in Microbial Ecology of the Gut. R. T. J. Clarke and T. Bauchop (ed.). Academic Press, London, UK.

Cobb-Vantress Inc., 2003. Product Focus. http://cobb-vantress.com/library/pdf/focus_product_1-03.pdf. Accessed July 12, 2004.

Cox, N. A., J. S. Bailey, and N. J. Stern, 2001. Effectiveness of an undefined mucosal competitive exclusion treatment to control *Salmonella* in turkeys during brooding. J. Appl. Poult. Res. 10:319-322.

Culter, G. C., 2002. Diseases of the chicken. Pages 473-542 in Commercial Chicken Meat and Egg Production. 5th Edition. D. D. Bell, W. D. Weaver Jr, (ed.), Kluwer Academic Publishers, Norwell, Massachusetts.

Danicke, S., W. Vahjen, O. Simon, and H. Jeroch, 1999. Effects of dietary fat type and xylanase supplementation to rye-based broiler diets on selected bacterial groups adhering to the intestinal epithelium, on transit time of feed, and on nutrient digestibility. Poult. Sci. 78:1292-1299.

Dalloul, R. A., H. S. Lillehoj, T. A. Shellem, and J. A. Doerr, 2003. Enhanced mucosal immunity against *Eimeria acervulina* in broilers fed a *Lactobacillus*-based probiotic. Poult. Sci. 82:62-66.

Denbow, D. M., 2000. Gastrointestinal anatomy and physiology. Pages 299-325 in Sturkie's Avian Physiology, 5th Edition. G. C. Whittow (ed.). Academic Press, San Diego, CA.

Duke, G., 1993. Avian digestion. Pages 428-436 in Dukes' Physiology of Domestic Animals, 11th edition. M J. Swenson and W. O. Reece, (ed.), Cornell University Press, Ithaca, NY.

Dutta, G. N., and L. A. Devriese, 1981. Sensitivity and resistance to growth promoting agents in animal lactobacilli. J. Appl. Bacteriol. 51:283-288.

Dutta, G. N., and L. A. Devriese, 1984. Observations on the *in vitro* sensitivity and resistance of Gram-positive intestinal bacteria of farm animals to growth promoting antimicrobial agents. J. Appl. Bacteriol. 56:117-123.

Engberg, R. M., M. S. Hedemann, T. D. Lesser, and B. B. Jensen, 2000. Effect of zinc bacitracin and salinomycin on intestinal microflora and performance of broilers. Poult. Sci. 79:1311-1319.

Estrada, A., D. C. Wilkie, and M. Drew, 2001. Administration of *Bifidobacterium bifidum* to chicken broilers reduces the number of carcass condemnations for cellulitis at the abattoir. J. Appl. Poult. Res. 10:329-334.

Eyssen, H., and P. De Somer, 1967. Effects of *Streptococcus faecalis* and a filterable agent on growth and nutrient absorption in gnotobiotic chicks. Poult. Sci. 46:323-333.

Fairchild, A. S., J. L. Grimes, M. J. Wineland, and F. T. Jones, 1998. Disk diffusion antimicrobial susceptibility tests against Escherichia coli isolates. Poult. Sci. 77(Suppl. 1): 94. (Abstract).

Fairchild, A. S., J. L. Grimes, F. T. Jones, M. J. Wineland, F. W. Edens, and A. E. Sefton, 2001. Effects of hen age, Bio-Mos,® and Flavomycin® on poult susceptibility to oral Escherichia coli challenge. Poult. Sci. 80:562-571.

Fanguy, R. C., L K. Misra, K. V. Vo, C. C. Blohowiak, and W. F. Krueger, 1980. Effect of delayed placement on mortality and growth performance of commercial broilers. Poult. Sci. 59:1215-1220.

Freter, R., E. Stauffer, D. Cleven, L. V. Holdeman, and W. E. C. Moore, 1983. Continuous flow cultures as in vitro models of the ecology of large intestinal flora. Infect. Immun. 39:666-675.

Fukata, T., Y. Hadate, E. Baba, and A. Arakawa, 1991. Influence of bacteria on Clostridium perfringens infections in young chicks. Avian Dis. 35:224-227.

Fuller, R., 1973. Ecological studies on the Lactobacillus flora associated with the crop epithelium of the fowl. J. Appl. Bacteriol. 36:131-139.

Fuller, R., 1977. The importance of lactobacilli in maintaining normal microbial balance in the crop. Br. Poult. Sci. 18:85-94.

Fuller, R., 1989. Probiotics in man and animals. J. Appl. Bacteriol. 66:365-378.

Fuller, R., 1992. History and development of probiotics. Pages 1-8 in Probiotics: The Scientific Basis. R. Fuller, (ed.), Chapman and Hall, London, U. K.

Fuller, R. and B. E. Brooker, 1974. Lactobacilli which attach to the crop epithelium of the fowl. Am. J. Clin. Nutr. 27:1305-1312.

Fuller, R., and A. Turvey, 1971. Bacteria associated with he intestinal wall of the fowl (*Gallus domesticus*). J. Appl. Bacteriol. 34:617-622.

Fuller, R., S. B. Houghton, and B. E. Brooker, 1981. Attachment of *Streptococcus faecium* to the duodenal epithelium of the chicken and its importance in colonization of the small intestine. Appl. Environ. Microbiol. 41:1433-1441.

Gleeson, T. M., S. Stavric, and B. Blanchfield, 1989. Protection of chicks against *Salmonella* infection with a mixture of pure cultures of intestinal bacteria. Avian Dis. 33:636-642.

Gong, J., R. J. Forester, H. Yu, J. R. Chambers, R. Wheatcroft, P. M. Sabour, and S. Chen, 2002. Molecular analysis of bacterial populations in the ileum of broiler chickens and comparison with bacteria in the cecum. Microbiol. Ecol. 41:171-179.

Goodwin, K., 1961. Effect of hatching egg size and chick size upon subsequent growth rate in chickens. Poult. Sci. 40:1408-1409.

Guan, L. L., K. E. Hagen, G. W. Tannock, D. R. Korver, G. M. Fasenko, and G. E. Allison, 2003. Detection and identification of *Lactobacillus* species in crops of broilers of different ages by using PCR-denaturing gradient gel electrophoresis and amplified ribosomal DNA restriction analysis. Appl. Environ. Microbiol. 69:6750-6757.

Guan, L. L., K. E. Hagen, T. L. Grayson, G. W. Tannock, D. R. Korver, G. M. Fasenko, and G. E. Allison, 2004. Detection of *Lactobacillus acidophilus* species in the gut of the chicken. Poster Presentation N147, 104th General Meeting of the American Society for Microbiology, New Orleans, LA

Guill, R. A., and K. Washburn, 1973. Relationship between hatch weight as a percentage of egg weight and feed conversion ratio in broiler chicks. Poult. Sci. 52:1641-1646.

Gupta, N. D., and K. S. Johar, 1975. Early body weight of five strains of White Leghorns. Br. Poult. Sci. 16:593-598.

Haddadin, M. S. Y., S. M. Abdulrahim, E. A. R. Hashlamoun, and R. K. Robinson, 1996. The effect of *Lactobacillus acidophilus* on the production and chemical composition of hen's eggs. Poult. Sci. 75:491-494.

Hager J. E., and W. L. Beane, 1983. Posthatch incubation time and early growth of broiler chickens. Poult. Sci. 62:247-254.

Hays, F. A., and E. W. Spear, 1952. Relation of age of parents to mortality and sex ratio of chicks at eight weeks. Poult. Sci. 31:792-795.

Hearn, P. J., 1986. Making use of small hatching eggs in an integrated broiler company. Br. Poult. Sci. 27:498.

Hill, K. J., 1976. The anatomy and general physiology of the alimentary tract. Pages 3-24 in Digestion in the fowl. K. N. Boorman, and B, M. Freeman (ed.). British Poultry Science Ltd., Edinburgh, UK.

Hofacre, C. L., T. Beacorn, S. Collett, and G. Mathis, 2003. Using competitive exclusion mannan-oligosaccharides and other intestinal products to control necrotic enteritis. J. Appl. Poult. Res. 12:60-64.

Hollister, A. G., D. E. Corrier, D. J. Nisbet, and J. R. Deloach, 1999. Effects of chicken-derived cecal microorganisms maintained in continuous culture on cecal colonization by *Salmonella typhimurium* in turkey poults. Poult. Sci. 78:546-549.

Howe, K., A. H. Linton, and A. D. Osborne, 1976. The effect of tetracycline on the coliform gut flora of broiler chickens with special reference to antibiotic resistance and o-serotypes of *Escherichia coli*. J. Appl. Bacteriol. 41:453-464.

Huang, M. K., Y. J. Choi, R. Houde, J. W. Lee, B. Lee, and X. Zhao, 2004. Effects of lactobacilli and an acidophilic fungus on the production performance and immune responses in broiler chickens. Poult. Sci. 83:788-795.

Humbert, F., G. Salvat, M. Morin, P. Colin, C. Lahellec, and G. Bennejean, 1989. La colonization spontanee de la muqueuse caecale du poulet exempt d'organismes pathogens specifies et l'implantation provoquee d'une flore de barriere chez le poulet conventionnel: etude au microscope electronique a baylage. Avian Pathol. 18:577-589.

Hume, M. E., L. F. Kubena, T. S. Edrington, C. J. Donskey, R. W. Moore, S. C. Ricke and D. J. Nisbet, 2003. Poultry digestive microflora biodiversity as indicated by denaturing gradient gel electrophoresis. Poult. Sci. 82:1100-1107.

Isolauri, E., Y. Sütas, P. Kankaanpää, H. Arvilommi, and S. Salminen, 2001. Probiotics: effects on immunity. Am. J. Clin. Nutr. 73(Suppl): 444S – 450S.

Jernigan, M. A., R. D. Miles, and A. S. Arafa, 1985. Probiotics in poultry nutrition - a review. Worlds Poult. Sci. 41:99-107.

Jerrett, S. A., and W. R. Goodge, 1973. Evidence for amylase in avian salivary glands. J. Morphol. 139:27-46.

Jin, L. Z., Y. W. Ho, M. A. Ali, N. Abdullah, and S. Jalaludin, 1996. Effect of adherent *Lactobacillus* spp. on *in vitro* adherence of salmonellae to the intestinal epithelial cells of chicken. J. Appl. Bacteriol. 81:201-206.

Jin, L. Z., Y. W. Ho, N. Abdullah, and S. Jalaludin, 1998. Growth performance, intestinal microbial populations, and serum cholesterol of broilers fed diets containing *Lactobacillus* cultures. Poult. Sci. 77:1259-1265.

Jin, L. Z., Y. W. Ho, M. A. Ali, N. Abdullah, and S. Jalaludin, 2000. Digestive and bacterial enzyme activities in broilers fed diets supplemented with *Lactobacillus* cultures. Poult. Sci. 79:886-891.

Jiraphocakul, S., T. W. Sullivan, and K. M. Shahani, 1990. Influence of a dried *Bacillus subtilis* culture and antibiotics on performance and intestinal microflora in turkeys. Poult. Sci. 69:1966-1973.

Jones, G. W., L. A. Richardson, and J. L. Vanden-Bosch, 1980. Phases in the interaction between bacteria and animal cells. Pages 211-219 in Microbial Adhesion to Surfaces. R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent (ed.) Ellis Horwood Ltd., Chinchester, U.K.

Knarreborg, A., R. M. Engberg, S. K. Jensen, and B. B. Jensen, 2002. Quantitative determination of bile salt hydrolase activity in bacteria isolated from the small intestine of chickens. Appl. Environ. Microbiol. 68:6425-6428.

Kosin, I. L., H. Abplanalp, J. Gutierrez, and J. S. Carver, 1952. The influence of egg size on subsequent early growth of the chick. Poult. Sci. 31:247-254.

Kubena, L. F., R. H. Bailey, J. A. Byrd, C. R. Young, D. E. Corrier, L. H. Stanker, and G. E. Rottinghaus, 2001. Cecal volatile fatty acids and broiler chick susceptibility to *Salmonella typhimurium* colonization as affected by aflatoxins and T-2 toxin. Poult. Sci. 80:411-417.

LaRagione, R. M., G. Casula, S. M. Cutting, and M. J. Woodward, 2001. *Bacillus subtilis* spores competitively exclude *Escherichia coli* O78:K80 in poultry. Vet. Microbiol. 79:133-142.

Lilly, D. M., and R. H. Stillwell, 1965. Probiotics: growth promoting factors produced by microorganisms. Science. 147:747-748.

Mattila-Sandholm, T., J. Mättö, and M. Saarela, 1999. Lactic acid bacteria with health claims – interactions and interference with gastrointestinal flora. Int. Dairy J. 9:25-35.

McClung, M. R., and R. M. Smith, 1949. Relationship between egg weight and growth rate of market chickens. Poult. Sci. 28:774.

McNaughton, J. L., J. W. Deaton, F. N. Reece, and R. L. Haynes, 1978. Effect of age of parents and hatching egg weight on broiler chick mortality. Poult. Sci. 57:38-44.

McReynolds, J. L., D. Y. Caldwell, E. T. Barnhart, J. R. Deloach, A. P. McElroy, R. W. Moore, B. M. Hargis, and D. J. Caldwell, 2000. The effect of in ovo or day-of-hatch subcutaneous antibiotic administration on competitive exclusion culture (PREEMPT™) establishment in neonatal chickens. Poult. Sci. 79:1524-1530.

Mead, G. C., 1997. Bacteria in the gastrointestinal tract of birds. Pages 216-240 in Gastrointestinal Microbiology, vol. 2. Gastrointestinal Microbes and Host Interactions. R. I. Mackie, B. A. White, and R. E. Isaacson (ed.), Chapman and Hall, New York, NY.

Mead, G. C., 2002. Factors affecting intestinal colonization of poultry by *Campylobacter* and role of microflora in control. Worlds Poult. Sci. 58:169-178.

Mead, G. C., and B. W. Adams, 1975. Some observations on the cecal microflora of the chick during the first two weeks of life. Br. Poult. Sci. 16:169-176.

Merritt, E. S., and R. S. Gowe, 1965. Post embryonic growth in relation to egg weight. Poult. Sci. 44:477-480.

Mohan, B., R. Kadirvel, A. Natarajan, and M. Bhaskaran, 1996. Effect of probiotic supplementation on growth, nitrogen utilization and serum cholesterol in broilers. Br. Poult. Sci. 37:395-401.

Moran, E. T. Jr., 1982. Large intestinal complex. Pages 177-198 in Comparative Nutrition of Fowl and Swine: The Gastrointestinal Systems. Office for Educational Practice, University of Guelph, Guelph, ON, Canada.

Morris, R. H., D. F. Hessels, and R. J. Bishop, 1968. The relationship between hatching egg weight and subsequent performance of broiler chickens. Br. Poult. Sci. 9:305-315.

Nisbet, D. J., D. E. Corrier, and J. R. DeLoach, 1993. Effect of mixed cecal microflora maintained in continuous culture and of dietary lactose on *Salmonella typhimurium* colonization in broiler chicks. Avian Dis. 37:528-535.

Nisbet, D. J., G. I. Tellez, V. K. Lowry, R. C. Anderson, G. Garcia, G. Nava, D. E. Corrier, and L. H. Stanker, 1998. Effect of a commercial competitive exclusion culture (Preempt) on mortality and horizontal transmission of *Salmonella gallinarum* in broiler chickens. Avian Dis. 42:651-656.

Nurmi, E., and M. Rantala, 1973. New aspects in *Salmonella* infections in broiler production. Nature. 241:210-211.

O'Neil, J. B., 1950. Relationship of chick size to egg size and its effect upon growth and mortality. Poult. Sci. 29:774(Abstract).

O'Neil, J. B., 1955. Percentage size of chick at hatching and its relationship to growth and mortality. Poult. Sci. 34:761-764.

Palmu, L., and I. Camelin, 1997. The use of competitive exclusion in broilers to reduce the level of *Salmonella* contamination on the farm and at the processing plant. Poult. Sci. 76:1501-1505.

Pascual, M., M. Hugas, J. I. Badiola, J. M. Monfort, and M. Garriga, 1999. *Lactobacillus salivarius* CTC2197 prevents *Salmonella enteritidis* colonization in chickens. Appl. Environ. Microbiol. 65:4981-4986.

Perdigon, G., S. Alvarez, M. Rachid, G. Aguero, and N. Gobbato, 1995. Immune system stimulation by probiotics. J Dairy Sci. 78: 1597-1606.

Pinchasov, Y., and Y. Noy, 1993. Comparison of post-hatch holding time and subsequent early performance of broiler chicks and turkey poults. Br. Poult. Sci. 34:111-120.

Pivnick, H. B., B. Blanchfield, and J. Y. D'Aoust, 1981. Prevention of *Salmonella* infection in chicks by treatment with fecal culture from mature chickens (Nurmi cultures). J. Food Prot. 44:909-913.

Pope, C. W., and P. J. Schaible, 1957. Association of egg weight and chick weight. Poult. Sci. 36:1149(Abstract).

Potter, L. M., L. A. Newborn, C. M. Parsons, J. R. Shelton, and J. S. Crawford, 1979. Effects of protein, poultry by-product meal, and dry *Lactobacillus acidophilus* culture additions to diets of growing turkeys. Poult. Sci. 58:1095 (Abstract).

Proudfoot, F. G., and H. W. Hulan, 1981. The influence of hatching egg size on the subsequent performance of broiler chickens. Poult. Sci. 60:2167-2170.

Proudfoot, F. G., H. W. Hulan, and K. B. McRae, 1982. Effect of hatching egg size from semi-dwarf and normal maternal meat parent genotypes on the performance of broiler chickens. Poult. Sci. 61:655-660.

Raibaud, P., 1992. Bacterial interactions in the gut. Pages 9-28 in Probiotics: The Scientific Basis. R. Fuller (ed.), Chapman and Hall, London, UK.

Reinhart, B. S., and G. I. Hurnik, 1984. Traits affecting the hatching performance of commercial chicken broiler eggs. Poult. Sci. 63:240-245.

Reuter, G., G. Klein, and M. Goldberg, 2002. Identification of probiotic cultures in food samples. Food Res. Int. 35:117-124.

Riddell, C., and X. M. Kong, 1992. The influence of diet on necrotic enteritis in broiler chickens. Avian Dis. 36:499-503.

Rowland, I. R., 1992. Metabolic interactions in the gut. Pages 29-53 in Probiotics: The Scientific Basis. R. Fuller (ed.), Chapman and Hall, London, UK.

Rubio, L. A., A. Brenes, I. Setien, G. de la Acuscion, N. Duran, and M. T. Cutuli, 1998. Lactobacilli counts in crop, ileum, and caecum of growing broiler chickens fed on practical diets containing whole or dehulled sweet lupin (*Lupinus angustifolius*) seed meal. Br. Poult. Sci. 39:354-359.

Salanitro, J. P., I. G. Fairchilds, and Y. D. Zgornicki, 1974. Isolation, culutre characterics, and identification of anaerobic bacteria from the chicken cecum. Appl. Microbiol. 27:678-687.

Salanitro, J. P., I. G. Blake, P. A. Muirhead, M. Maglio, and J. R. Goodman, 1978. Bacteria isolated from the duodenum, ileum and cecum of young chicks. Appl. Environ. Microbiol. 35:782-790.

Sarra, P. G., L. Morelli, and V. Bottazzi , 1992. The lactic acid microflora of fowl. Pages 3-19 in The Lactic Acid Bacteria in Health and Disease. B. J. B. Wood (ed.), Elsevier Applied Science, London, UK.

Savage, D. C., 1977. Interactions between the host and its microbes. Pages 277-310 in Microbial Ecology of the Gut. R. T. J. Clarke, and T. Bauchop (ed.). Academic Press. London, UK.

Schneitz, C., T. Kiiskinen, V. Toivonen, and M. Nasi, 1998. Effect of Broilact on the physiochemical conditions and nutrient digestibility in the gastrointestinal tract of broilers. Poult. Sci. 77:426-432.

Seuna, E., and E. Nurmi, 1979. Therapeutical trials with antimicrobial agents and cultured cecal microflora in *Salmonella infantitis* infections in chickens. Poult. Sci. 58:1171-1174.

Shanawany, M. M., 1987. Hatching weight in relation to egg weight in domestic birds. Worlds Poult. Sci. 43:107-115.

Sheldon, B. W., and E. O. Essary, 1982. Effect of antibiotics on intestinal microflora and flavor of broiler meat. Poult. Sci. 61:280-287.

Silva, E. N., G. H. Snoeyenbos, O. M. Weinack, and C. F. Smyser, 1981. The influence of native gut microflora on the colonization and infection of *Salmonella gallinarum* in chickens. Avian Dis. 25:68-73.

Sinclair, R. W., F. E. Robinson, and R. T. Hardin, 1990. The effects of parent age and posthatch treatment on broiler performance. Poult. Sci. 69:526-534.

Sklan, D., S. Heifetz, and O. Halevy, 2003. Heavier chicks at hatch improves marketing body weight by enhancing skeletal muscle growth. Poult. Sci. 82:1778-1786.

Skoglund, W. C., K. C. Seegar, and A. T. Ringrose, 1952. Growth of broiler chicks hatched from various sized eggs when reared in competition with each other. Poult. Sci. 31:796-799.

Smith, H. W., 1965. The development of the flora of the alimentary tract in young animals. J. Pathol. Bacteriol. 90:495-513.

Snoeyenbos, G. H., O. M. Weinack, and C. F. Smyser, 1978. Protecting chicks and poults from Salmonellae by oral administration of "normal" gut microflora. Avian Dis. 22:273-287.

Soerjadi, A. S., R. Rufner, G. H. Snoeyenbos, and O. M. Weinack, 1982. Adherence of Salmonellae and native gut microflora to the gatrointestinal mucosa of chicks. Avian Dis. 26:576-584.

Soerjadi-Liem, A. S., G. H. Snoeyenbos, and O. M,. Weinack, 1984. Comparative studies on competitive exclusion of three isolates of *Campylobacter fetus* subsp. *jejuni* in chickens by native gut microflora. Avian Dis. 28:139-146.

Sperti, G. S., 1971. Probiotics. Avi Publishing Co. West Point, CT.

Stavric, S., T. M. Gleeson, B. Blanchfield, and H. Pivnick, 1985. Competitive exclusion of *Salmonella* from newly hatched chicks by mixtures of pure bacterial cultures isolated from fecal and cecal contents of adult birds. J. Food Prot. 48:778-782.

Stavric, S., T. M. Gleeson, and B. Blanchfield, 1991. Effect of avian intestinal microflora possessing adhering and hydrophobic properties on competitive exclusion of Salmonella typhimurium from chicks. J. Appl. Bacteriol. 12:414-421.

Tannock, G. W., 1998. Studies of the intestinal microflora: a prerequisite for the development of probiotics. Int. Dairy J. 8:527-533.

Tannock, G., 2001. Molecular assessment of intestinal microflora. Am. J. Clin. Nutr. 73:410S-414S.

Tindell, L. D., and D. R. Morris, 1964. The effects of egg weight on subsequent broiler performance. Poult. Sci. 43:534-539.

Tortuero, F., 1973. Influence of the implantation of *Lactobacillus acidophilus* in chicks on the growth, feed conversion, malabsorption of fat syndrome and intestinal flora. Poult. Sci. 52:197-203.

Tullett, S. G., and F. G. Burton, 1982. Factors affecting the weight and water status of the chick at hatch. Br. Poult. Sci. 23:361-369.

Watkins, B. A., and F. H. Kratzer, 1983. Effect of oral dosing of *Lactobacillus* strains on gut colonization and liver biotin in broiler chicks. Poult. Sci. 62:2088-2094.

Watkins, B. A., and F. H. Kratzer, 1984. Drinking water treatment with a commercial preparation of a concentrated *Lactobacillus* culture for broiler chickens. Poult. Sci. 63:1671-1673.

Watkins, B. A., B. F. Miller, and D. H. Neil, 1982. *In vivo* inhibitory effects of *Lactobacillus acidophilus* against pathogenic *Escherichia coli* in gnotobiotic chicks. Poult. Sci. 61:1298-1308.

Weinack, O. M., G. H. Snoeyenbos, C. F. Smyser, and A. S. Soerjadi, 1982. Reciprocal competitive exclusion of *Salmonella* and *Escherichia coli* by native intestinal microflora of the chicken and turkey. Avian Dis. 26:585-595.

Wierup, M., M. Wold-Troell, E. Nurmi, and M. Häkkinen, 1988. Epidemiological evaluation of the *Salmonella*-controlling effect of a nationwide use of a competitive exclusion culture in poultry. Poult. Sci. 67:1026-1033.

Wiley, W. H., 1950. The influence of egg weight on pre-hatching and post-hatching growth rate in the fowl. II. Egg weight-chick weight ratios. Poult. Sci. 29:595-604.

Wyatt, C. L., W. D. Weaver Jr., and W. L. Beane, 1985. Influence of egg size, eggshell quality, and post-hatch holding time on broiler performance. Poult. Sci. 64:2049-2055.

Yeo, J., and K. I. Kim, 1997. Effect of feeding diets containing an antibiotic, a probiotic, or yucca extract on growth and intestinal urease activity in broiler chicks. Poult. Sci. 76:381-385.

Zulkifli, I., N. Abdullah, N. M. Azrin, and Y. W. Ho, 2000. Growth performance and immune response of two commercial broiler strains fed diets containing *Lactobacillus* cultures and oxytetracycline under heat stress conditions. Br. Poult. Sci. 41:593-597.

# 2. INVESTIGATING THE EFFECTS OF COMMERCIAL PROBIOTICS ON CHICK QUALITY AND BROILER PRODUCTION EFFICIENCY

## INTRODUCTION

Over the past several decades, the physiological stresses that have been placed on broilers in commercial production have increased. This increased stress is the result of practices employed in modern broiler production such as processing at the hatchery and high stocking densities (Pinchasov and Noy, 1993). Genetic selection for faster, more efficient growth may also place increased physiological stress on broilers. This is evidenced by a reduced immune function in modern broilers versus older genetic stock (Qureshi and Havenstein, 1994)

Chick viability and broiler growth are influenced by breeder flock age, with younger breeder flocks typically producing broiler chicks of poorer quality (Sinclair *et al.,* 1990). Younger breeders lay smaller eggs, which produce smaller chicks (Hays and Spear, 1952; McNaughton *et al*., 1978; Wyatt *et al*., 1985). These smaller chicks are associated with poorer quality (Sinclair *et al*., 1990), higher mortality (McNaughton *et al*., 1978), and lower market body weights (Morris *et al.,* 1968; Sklan *et al*., 2003). The combination of environment, parental age, and genetic factors can negatively impact early chick viability.

These increased stressors may weaken immune function, and thus predispose broilers to colonization of the gastrointestinal tract (GIT) by bacterial pathogens or other unfavorable microorganisms (Barnes, 1979; Hume *et al*., 2003). These microorganisms can include bacteria such as *Salmonella* and *Campylobacter* species, which pose a threat to food safety. Another bacterial pathogen, *Clostridium perfringens*, which is the causative agent of necrotic enteritis (NE), poses a threat to broiler health, thus reducing production efficiency and increasing mortality (Culter, 2002).

Since the mid 1950's the administration of sub-therapeutic doses of antibiotics throughout the production period has been the main approach to guard against the intestinal colonization of poultry by pathogenic bacteria. However, there are negative consequences associated with this method of disease control, the primary one being antibiotic resistance (Howe *et al.*, 1976; Estrada *et al.*, 2001). Consumers are also less willing to accept the use of antibiotics in animal agriculture than they have been in the past. Because of this, governments have imposed stricter regulations regarding the appropriate uses of antibiotics in animal agriculture. Poultry producers are also becoming more concerned with the overuse of antibiotics, since as more pathogens develop resistance to antibiotics, the effectiveness of the antibiotics available to producers to fight disease outbreaks is reduced.

The use of probiotics may provide an alternative to the administration of antibiotics to prevent colonization of the GIT by microorganisms pathogenic to birds. In addition, probiotics may prevent the GIT establishment of microorganisms that are commensal to the bird, but are human pathogens and present a threat to food safety if introduced into the food supply. Probiotics, also referred to as direct-fed viable microbial products, consist of live microbial cultures that are isolated from the GIT of humans or animals. Commercially produced probiotic products are usually species specific, with products intended for use in chickens comprised of bacterial species which would have been isolated from the GIT of chickens.

Microbial populations within the GIT develop very quickly after hatching (Guan *et al.*, 2004). Contact with microorganisms on the the eggshell (Coates and Fuller, 1977) or in feed (Smith, 1965; Barnes, 1972; Mead and Adams, 1975; Jones and Richardson, 2004) results in the initial microbial colonization of the GIT. It is during this early period, when a GIT microflora has not yet been established, that the chick is most vulnerable to colonization by pathogens such as *C. perfringens* or microorganisms such as *Salmonella* species, which are commensal in chickens but pathogenic to humans if consumed in contaminated meat.

The establishment of a healthy GIT microflora in newly hatched broiler chicks is crucial to preventing the colonization of the GIT by pathogenic microorganisms. It is thought that probiotics act to promote the development of a healthy GIT microflora (Blankenship *et al.*, 1993; Chambers and Lu, 2002). If a GIT microflora composed of bacterial species that are beneficial to the bird can be established, the colonization of pathogenic bacteria in the GIT can be avoided, even when the bird is eventually exposed to these microorganisms in the environment (Blankenship *et al.*, 1993; Palmu and Camelin, 1997; Kubena *et al.*, 2001; Chambers and Lu, 2002).

In traditional production systems, more than 40 years ago, the chicks were hatched and reared with the hen, and thus exposed to the feces of mature birds immediately after hatching. The feces contained the same bacterial species naturally present in the GIT of the hen, thus when the feces were ingested by the chick these bacterial species were able to colonize the chick's GIT. It is believed that colonization of the GIT with bacterial species normally present in the commensal microflora inhibits the ability of gastrointestinal pathogens and unfavorable microorganisms to colonize the chick's GIT. This is accomplished through competitive exclusion (Nurmi and Rantala, 1973; Nisbet *et al*., 1993), and the change in pH caused by lactic acid production (Fuller, 1977; Chateau *et al*., 1993).

Past research has shown that administering probiotics can provide the same protection as the commensal GIT microflora (Nurmi and Rantala, 1973; Pascual *et al*., 1999; Kubena *et al*., 2001; LaRagione *et al*., 2001). Improvements in the weight gains of broilers (Nurmi and Rantala, 1973), feed conversion ratio (FCR) (Jin *et al*., 1998), and food safety, through a reduction in the numbers of pathogenic bacteria colonizing the GIT (Chambers and Lu, 2002) have been demonstrated. Past research indicates that effective probiotic products may provide a viable alternative to antibiotic use in broiler production.

Probiotic products can be divided into two main categories: simple and complex. Simple probiotics are composed of only a few bacterial species, which are generally lactobacilli. Complex defined probiotics are bacterial cultures containing as many as 30 different bacterial species. Administering complex probiotics or dosing chicks with the undefined cecal contents of an adult chicken seems to provide more protection than doses of a single bacterial strain or a combination of only a few strains (Baba *et al*., 1991; Nisbet *et al*., 1993). However, the benefits offered by probiotics vary to a large extent based on the bacterial species, and even the particular strains of a given species included in the probiotic preparation (Schleifer, 1985). Other factors such as the microflora status of the bird, the method of administration, the age of the bird at the time of administration, and exposure to stressors, such as feed and water withdrawal or temperature stress, can also influence the efficacy of a probiotic preparation (For an extensive review, see Mead, 2000).

The Canadian Food Inspection Agency (CFIA) is the branch of the Canadian government that licenses veterinary biologics, including probiotics, for use in agricultural animals. Any veterinary biologic product that is approved for commercial use in Canada is first evaluated by the CFIA for purity, potency, safety, and efficacy (CFIA, 2001).

At the present time only two live microbial products are approved for use in poultry species in Canada: Interbac®[3] and Pro-Avi®[3]. Interbac® can be applied either in the drinking water or as a spray at the time of hatch. The bacterial species included, as listed by the manufacturer, are *Lactobacillus acidophilus*, *L. bifidus*, and *Streptococcus faecalis* (now reclassified as *Enterococcus faecalis*). Pro-Avi®, is a powdered feed additive which is administered to broilers throughout the entire production period. The manufacturer lists *L. acidophilus*, *S. faecalis* (now reclassified as *E. faecalis*), and bifidobacteria, (no specific species identified) as the bacteria included in this product.

The objectives of this experiment were to examine the efficacy (in broiler chickens) of the only two commercially available probiotics approved for use in poultry in Canada, and to determine if the effectiveness of these products in broilers varied with breeder flock age. It was hypothesized that each of the probiotic treatments would result in improved chick viability, increased weight gains, and improved feed conversion compared to that of the broilers not administered the probiotics. It was also anticipated that the probiotic treatments would have a greater impact on the performance of broilers from a young breeder flock as opposed to broiler chicks produced by an old breeder flock.

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[3] Intervet Canada, Inc., 250 Water Street, Whitby, ON, Canada, L1N 9T5.

## MATERIALS AND METHODS

The experimental protocol was approved by the Faculty of Agriculture, Forestry and Home Economics Animal Policy and Welfare Committee at the University of Alberta, in accordance with the guidelines set forth by the Canadian Council on Animal Care (1993).

### Incubation and Hatching

Twenty-five hundred Hubbard Hi-Y hatching eggs were obtained from a single commercial broiler breeder flock at each of three flock ages: 28, 43, and 57 wk of age. Any cracked eggs or eggs weighing less than 52 g were not used in the experiment. All settable eggs were weighed, numbered, and randomly divided into groups of 18 eggs for the purpose of statistical replication. Each group of 18 eggs was randomly placed within a 5000 egg capacity Jamesway[4] single stage setter and incubated for 18 d at a dry bulb temperature of 37.5°C and a wet bulb temperature of 29.4°C.

At seven d of incubation all eggs were removed from the setter and candled. Any eggs thought to contain non-viable embryos were broken open to assess fertility, and if fertile, the approximate day of embryonic death. At 18 d of incubation the eggs were removed from the setter, individually weighed, and transferred to a 5000 egg capacity Jamesway hatcher where they were incubated for an additional 3.5 d at a dry bulb temperature of 35.2°C and a wet bulb temperature of 29.4°C.

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[4] Jamesway Incubator Company Inc., Cambridge, ON, Canada, N1R 7L3.

**Broiler Production Period**

After 21.5 d of incubation, all hatched chicks were counted, and chick quality was visually assessed according to commercial hatchery standards. All unhatched eggs were broken open to determine the approximate day of embryonic death. Embryonic mortality was grouped into three stages: early (1 to 7 d), mid (8 to 14 d), and late (15-21 d). All chicks deemed to be saleable were individually weighed, neck tagged[5], and randomly allocated to one of four treatment groups: 1) control (CON) - no probiotics administered, 2) Interbac® water (IBW) - Interbac® administered in distilled drinking water as directed by the manufacturer at 1, 2, 19, and 20 d of age, 3) Interbac® spray (IBS) - Interbac® administered as a spray at hatch as directed by the manufacturer, 4) Pro-Avi® Feed (PAF) – Pro-Avi® administered as a feed additive (0.5 g Pro-Avi®/ 1 kg feed) throughout the production period.

Chicks allocated to each of the treatment groups were randomly placed at a stocking density of 0.07 $m^2$/bird (0.76 $ft^2$/bird) into two isolated environmental chambers, with each chamber divided into two pens. The number of chicks placed in each pen varied between trials (28 wk of breeder flock age – 110 chicks/pen; 43 wk of breeder flock age - 124 chicks/pen; 57 wk of breeder flock age - 104 chicks/pen) due to differences in the number of saleable chicks hatched, but stocking density was held constant across the three trials by partitioning off the excess space as necessary. Each environmental chamber was equipped with an individual ventilation system vented to the exterior of the building, individual access doors and disinfectant boot dips (filled with Virkon[6]). Separate coveralls and boots were allocated to each chamber to prevent the transmission of microorganisms between chambers and thus between the treatment groups. The chambers were fumigated using formaldehyde prior to the placement of straw in the pens. Once the birds were placed in their respective chambers, the probiotic treatments were administered. All probiotics were administered according to the manufacturer's directions. Only the birds in the IBW treatment were provided with distilled water, and only for the time during which the treatment was administered. All other treatment groups received chlorinated, city-supplied drinking water.

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[5] Heartland Animal Health Inc., 363 Highway 32, Fair Play MO, 65649.

[6] Antec International, Chilton Industrial Estate, Sudbury, Suffolk, U.K., C010 2XD

The broilers were reared on straw litter for six wk. They were fed a standard broiler starter ration (Tables 2-1 and 2-2) for the first three wk, and a standard broiler grower ration (Tables 2-1 and 2-2) for the remaining three wk of the production period. A coccidiostat (Amprol) was included in the feed, but no other antimicrobial agents were administered.

The viability of the probiotic cultures, and their persistence in the water for the IBW treatment and in the feed for the PAF treatment were confirmed to remain at or above levels specified by the manufacturer throughout the six wk broiler production period. This was accomplished by plating serial dilutions of the feed or water sample on Man Rogosa Sharp (MRS) agar to selectively culture lactic acid bacteria. The number of colony forming units (CFU) was then calculated and compared to the manufacturer's guaranteed minimum number of CFU (L. Guan, personal communication).

A sample of 30 broilers, nearest the average chick weight from each pen, (120 broilers per treatment group), were identified and individually weighed at 7, 14, 21, 28, and 35 d of age. Feed consumption in each pen was measured on a weekly basis. At 42 d of age, all broilers were individually weighed prior to shipping. Mortality in each pen was recorded on a daily basis, and all birds that died during the production period were necropsied at the conclusion of the three trials.

### Statistical Analysis

All data were analyzed using the general linear model (GLM) procedure of SAS® (SAS Institute, 1999). All percentage data were transformed using arc sine transformation prior to analysis. Significance was assessed at $P<0.05$. Where the model indicated significance, the means were separated using the P-DIFF procedure of SAS®.

## RESULTS & DISCUSSION

### Egg Weights and Weight Loss

Average egg weights at setting increased significantly as the breeder flock aged (Table 2-3). This is in agreement with previous research (McNaughton *et al.*, 1978; Wyatt *et al.*, 1985). Average egg weights at transfer and percentage egg weight loss at the time of transfer followed the same trend as egg weight. Percent weight loss increased as the flock aged. This was expected because as egg size increases, shell thickness decreases, resulting in increased eggshell conductance (Ar *et al.*, 1974) and thus greater moisture loss.

### Fertility, Hatchability, Embryonic Mortality, and Culled Chicks

Because probiotic treatments were imposed after hatching, hatch characteristics and egg weights will only be discussed with regard to breeder flock age. Fertility was significantly different between all three flock ages, with the highest fertility at 43 wk, and the lowest at 57 wk (Table 2-4). Hatchability of all eggs set and hatchability of fertile eggs followed the same pattern as fertility, with eggs from the oldest flock having the poorest hatchability. There were also differences in early, mid, and late embryonic mortality due to flock age. Early embryonic mortality was lowest in the 43 wk old flock compared to the 28 and 53 wk old flocks, which did not differ from one another. Both mid and late embryonic mortality was highest in the 57 wk old flock compared to the 28 and 43 wk old flocks, which did not differ from each other. There were also significantly higher percentages of culled chicks in both the 28 and 57 wk old flocks compared to the 43 wk old flock (Table 2-4). This indicates that both the youngest and oldest flocks had poorer overall chick quality than did the chicks from the breeder flock nearest peak production.

This is not consistent with some previous research showing that the percentage of saleable chicks hatched is higher in eggs from younger breeder flocks (McNaughton *et al.*, 1978). However, the results in the present study do agree with previous findings of poor hatchability in extremely small and large eggs from extremely young and old breeder flocks, respectively (Lerner and Gunns, 1952; Morris *et al.*, 1968).

### Body Weights and Weight Gains

***Effect of Breeder Flock Age.*** Chick weight increased significantly as the breeder flock aged (Table 2-5). This was expected, since past research has determined that smaller eggs from younger breeder flocks produce smaller chicks (McNaughton *et al.*, 1978; Wyatt *et al.*, 1985). Chick weight is traditionally used as an evaluation of chick quality; lighter broiler chicks are usually of poorer quality, having lower weight gains and final market weights (Sklan *et al.*, 2003).

At both one and two wk of age the broilers from the 28 wk old breeder flock were significantly lighter than those from either 43 or 57 wk of breeder flock age. At three wk there were significant differences between the broilers from each breeder flock age, with broiler weights increasing as breeder flock age increased. From four wk of age until the end of the production period, the broilers from the 28 wk old breeder flock were significantly lighter than the broilers from either the 43 or 57 wk old breeder flocks, which did not differ from one another.

There were also significant differences in weight gains among the chicks from different breeder flock ages (Table 2-6). Up to five wk of age, broilers from the 28 wk old flock consistently had significantly lower weight gains than broilers produced when the flock was 43 and 57 wk of age. During the second and third wk, the body weight gains were significantly different between all breeder flock ages, with weight gain increasing with breeder flock age. The overall weight gain was lowest in broilers from the 28 wk old flock compared to the other flock ages, which did not differ from one another.

These results were expected, since it is well documented that as breeder flock age increases, so does egg size (McNaughton *et al.*, 1978). Larger eggs from older breeder flocks subsequently produce larger chicks, and broilers with heavier final body weights (Merritt and Gowe, 1965; Morris *et al.*, 1968; Sklan *et al.*, 2003).

***Effect of Probiotic Treatments.*** There were significant differences in chick weight at placement between treatments (Table 2-5). However, the weight differences were less than 0.25 g. Although statistically different, the differences were not deemed to be biologically significant. Because of the differences in chick weight between the probiotic treatments at placement, the weights and weight gains for subsequent weeks were analyzed as a covariate analysis. By one wk of age, there were no significant differences in broiler weight between the probiotic treatments, and there were no significant differences in broiler weights between treatments throughout the rest of the production period (Table 2-5).

There were no differences in weekly weight gains between probiotic treatments from the first to the fifth wk (Table 2-6). However, the weight gain in the sixth wk was significantly higher in the IBW, IBS, and PAF treatments compared to the CON treatment. This indicates that administering the probiotics may improve weight gain in the final week of production. However, this did not result in significant differences in final market body weights. The overall weight gains over the six week grow out period did not differ between probiotic treatments (Table 2-6).

The available body of literature offers a variety of conflicting results concerning the efficacy of probiotics for increasing body weights and weight gains in broilers. Whereas undefined, or complex defined probiotic cultures have generally improved weight gains and market body weights (Nurmi and Rantala, 1973; Tortuero, 1973) simple defined probiotics have been used with mixed results (Watkins and Kratzer, 1983; Watkins and Kratzer, 1984; Yeo and Kim, 1997; Jin *et al.*, 1998; Estrada *et al.*, 2001; Hofacre *et al.*, 2003). It is thus possible that in the present study, the low number of bacterial strains in the probiotic products may have limited their efficacy in improving body weights and weight gains.

***Effect of Breeder Flock Age x Probiotic Treatment.*** Only chick weight at placement was significantly influenced by the interaction between the main effects of breeder flock age and probiotic treatment (Table 2-5). For this reason, subsequent body weights were analyzed as a covariate analysis to account for these initial differences in chick weight. There was no effect of the interaction on body weights (Table 2-5) or weight gains (data not shown) for the entire production period. This was contrary to the hypothesis, since it was anticipated that the probiotics would benefit the broilers from the younger breeder flock to a greater extent than the broilers from the peak and older breeder flocks.

### Feed Conversion

***Effect of Breeder Flock Age.*** During the first wk, the broilers from the 28 wk old breeder flock had a significantly higher FCR than did the broilers from either of the other two older breeder flock ages (Table 2-7). This changed in the second wk, with the broilers from the 43 wk old breeder flock having a significantly higher FCR than the broilers from the 28 and 57 wk old breeder flocks. During weeks three, five, and six there were no differences in FCR due to breeder flock age. At four wk, the broilers from the 43 wk old breeder flock had a lower FCR compared to the broilers from the 28 wk old flock; neither was significantly different from the broilers from the 57 wk old breeder flock. Over the entire production period, the broilers from the 28 wk old flock had the best FCR compared to the broilers from the 57 wk old flock. The FCR of the broilers from the 43 wk old flock did not differ from either of the other two flock ages. This agrees with previous findings that smaller chicks exhibit a better FCR than larger chicks (O'Neil, 1955; Morris *et al.*, 1968; Proudfoot and Hulan, 1981; Wyatt *et al.*, 1985; Hearn, 1986). It is unclear whether this is an effect of breeder flock age, or simply of chick size.

***Effect of Probiotic Treatments.*** There was no effect of any of the probiotic treatments on the FCR at any point during the production period, or on the overall FCR over the entire production period (Table 2-7). Past studies using simple, defined probiotics have found improvements in broiler FCR (Jin *et al.*, 1998; Zulkifli *et al.*, 2000). However, others have not noted differences in FCR between probiotic treated birds and untreated control birds (Watkins and Kratzer, 1983; Watkins and Kratzer, 1984; Estrada *et al.*, 2001; Huang *et al.*, 2004).

These varying results may be due to differences in the bacterial strains used in the above mentioned studies and the origins of these strains. Since in most studies no information is provided as to whether the strain used was isolated from poultry, it is not possible to assess whether it is host specific and would be able to attach to the GIT epithelial cells (Jin *et al.*, 1998; Cox *et al.*, 2001). In the present study, no information as to the origin of the bacterial strains used was provided by the manufacturer, so it is impossible to assess whether this may play a role.

***Effect of Breeder Flock Age x Probiotic Treatment.*** It was anticipated that the probiotic treatments would result in greater improvements in FCR in chicks from a young breeder flock compared to chicks from older breeder flocks, but this was not shown to be the case. There was no significant effect of the interaction between treatment and breeder flock age on FCR overall, or at any point during the six wk grow out period (data not shown).

**Broiler Mortality**

***Effect of Breeder Flock Age.*** Breeder flock age had a significant effect on first wk broiler mortality and second wk cumulative mortality. Broilers produced by the 57 wk old breeder flock had significantly higher mortality than broilers produced from the 28 and the 43 wk old breeder flocks, which did not differ from one another (Table 2-8). From three wk of age until the end of the production period, there were no significant differences in cumulative broiler mortality due to breeder flock age. Despite the fact that the younger flock produced smaller chicks with lower weight gains, there was no significant difference in total mortality between the broilers from the young flock and broilers produced at the two older flock ages. This is in contrast to previous research showing that smaller chicks experience a higher mortality rate (McClung and Smith, 1949; O'Neil, 1950; Hays and Spear, 1952; McNaughton *et al.*, 1978; Wyatt *et al.*, 1985; Hearn, 1986). This may be due to the fact that while the broilers were all reared in straw floor pens, the rearing conditions were very sanitary, so chicks from the younger breeder flock may not have faced as many pathogen challenges as they would in a commercial situation.

***Effect of Probiotic Treatment.*** There was no effect of probiotic treatment on cumulative mortality from one to five wk of age (Table 2-8). However, the probiotics treatments did have an effect on six wk cumulative broiler mortality. Both the IBW and the IBS treatments had higher mortality than the PAF treatment. However, none of the probiotic treatments (IBW, IBS, or PAF) were significantly different from the control. This result was not unexpected, since there appears to be no evidence that probiotics, either complex or simple are capable of reducing broiler mortality (Bilgili and Moran, 1990; Palmu and Camelin, 1997; Jin *et al*., 1998; Estrada *et al*., 2001), despite their efficacy in reducing the number of pathogens colonizing the GIT (Blankenship *et al*., 1993; Palmu and Camelin, 1997; Chambers and Lu, 2002).

***Effect of Breeder Flock Age x Probiotic Treatment.*** There was no effect of the interaction between the two main factors of breeder flock age and probiotic treatment on broiler mortality at any point during the production period (data not shown).

### Incidence of Necrotic Enteritis (NE)

In some of the broiler pens at each of the breeder flock ages there were cases of NE. While there were sub-clinical cases of NE (resulting in possible growth depression, but low or no mortality) in several pens at each of the three trials, there were clinical outbreaks (resulting in a rapid rise in the rates of morbidity and mortality) in one CON pen in the trial at 43 wk of breeder flock age (during the third wk of the broiler production period) and in one PAF and one IBS pen in the trial at 57 wk of breeder flock age (during the fourth week of the broiler production period). The clinical outbreaks necessitated the treatment of the affected pens with therapeutic levels of antibiotics administered in the drinking water. Subsequently, the decision was made to completely remove all the data collected from these pens from the data analysis. In the remaining pens there were no significant differences in the incidence of NE between probiotic treatments or flock ages, as confirmed by post mortem examination of all birds that died during the trials.

Since NE infects the bird by colonizing the small intestine (Culter, 2002), the incidence of NE in all treatment groups provides further data indicating that the probiotic products investigated in this research did not protect the GIT from colonization by pathogenic microorganisms such as *C. perfringens*, the causative agent of NE. This is contrary to past research which has shown that either a simple probiotic culture, or an undefined culture consisting of adult cecal material is able to prevent colonization of the GIT by *C. perfringens* (Fukata *et al.*, 1991; Hofacre *et al.*, 2003), reduce the toxicity of *C. perfringens* (Fukata *et al.*, 1991), and reduce NE associated mortality (Hofacre *et al.*, 2003). In general, it appears that most probiotics, both simple and complex, are more effective in competitively excluding *Salmonella* than other potentially harmful bacteria (Blankenship *et al.*, 1993; Pascual *et al.*, 1999; Kubena *et al.*, 2001; Chambers and Lu, 2002). No microscopic or microbiological analysis was performed on broiler GIT samples in the present study, so no data with respect the colonization of the broiler GIT by pathogens was collected.

The diet used in the present study was wheat-based, as is commonly the case in the prairie region of Canada. This may also have increased the likelihood of NE infection, since diets high in wheat have been linked to a higher incidence of NE than corn based diets (Riddell and Kong, 1992; Annett *et al.*, 2002).

## Summary and Conclusions

It was initially hypothesized that the broilers in the IBW, IBS, and PAF probiotic treatments would all have higher weight gains and body weights, lower mortality, and better FCR than the broilers in the control group. This was not shown to be the case. It was also hypothesized that there would be a effect of the probiotic treatments on broiler performance in chicks produced by a young breeder flock, since young flocks generally produce poorer quality chicks. It was determined that both the young (28 wk) and old (57 wk) breeder flocks produced poorer quality chicks than the chicks produced from the near peak-aged flock (43 wk). This was based on the fact that the peak-aged flock had higher hatchability, and a lower percentage of culled chicks than either the younger or older breeder flocks. During the broiler grow out period, the 43 and 57 wk old breeder flocks produced broilers with higher overall weight gains, despite the fact that the broilers from the younger flock had a lower FCR. There was no effect of the interaction between treatment and breeder flock age on any production parameter.

Previous research has shown probiotics can be capable of improving broiler weight gains and market body weights (Nurmi and Rantala, 1973; Mohan *et al.*, 1996; Jin *et al.*, 1998). However, the probiotics tested in the current trial did not result in improvements in broiler production efficiency over the control group. This result occurred despite the fact that the theoretical doses (on a colony forming units (CFU) per bird basis) were above the number of CFU guaranteed by the manufacturer and above the minimum effective dose of $1 \times 10^5$ CFU/bird proposed by Pascual *et al.* (1999) (See Appendix). However, the lack of significant effect of the probiotics investigated in the present study may be due to inconsistencies between the microbiological composition of the products as described by the manufacturer, and the microbiological composition revealed by a laboratory analysis conducted in concert with this thesis (See Appendix).

**TABLE 2-1. Ingredient composition of the standard broiler starter and grower rations fed in the present experiment**

| Ingredient | Starter Diet Inclusion Rate (g/kg) | Grower Diet Inclusion Rate (g/kg) |
|---|---|---|
| Wheat | 621.0 | 535.3 |
| Corn | 18.7 | 148.5 |
| Soybean Meal | 245.1 | 218.7 |
| Canola Meal | 16.0 | 0.0 |
| Canola Oil | 55.0 | 56.0 |
| Dicalcium Phosphrous | 14.8 | 11.1 |
| Limestone | 13.0 | 14.0 |
| Choline Chloride | 5.0 | 5.0 |
| Broiler Vitamin Pre-Mix | 5.0 | 5.0 |
| Salt | 4.0 | 3.5 |
| DL-Methionine | 1.6 | 0.8 |
| L-Lysine | 0.4 | 1.1 |
| Amprol | 0.5 | 0.5 |
| Avizyme 1302 | 0.5 | 0.5 |

**TABLE 2-2. Nutrient composition of the standard broiler starter and grower rations fed in the present experiment**

| Diet Component | Starter Diet Compostion | Grower Diet Compostion |
|---|---|---|
| Protein (%) | 21.35 | 19.6 |
| ME (kcal/kg) (%) | 3000 | 3098 |
| Linoleic Acid (%) | 1.74 | 2.02 |
| Fat (%) | 7.43 | 7.48 |
| Calcium (%) | 0.93 | 0.86 |
| Available Phosphrous (%) | 0.42 | 0.34 |
| Chloride (%) | 0.29 | 0.25 |
| Sodium (%) | 0.19 | 0.17 |
| Lysine (%) | 1.02 | 0.97 |
| Methionine (%) | 0.46 | 0.37 |
| Cystine (%) | 0.38 | 0.34 |

**TABLE 2-3. Egg weights at setting and transfer, and percent weight loss of eggs produced by a breeder flock at three ages**

| Breeder Flock Age | Set Weight (g) | Transfer Weight (g) | Weight Loss[1] (%) |
|---|---|---|---|
| 28 wk | 55.8 ± 0.1 $^{c}$<br>2250[2] | 49.4 ± 0.1 $^{c}$<br>1998 | 11.4 ± 0.1 $^{c}$<br>1998 |
| 43 wk | 61.9 ± 0.1 $^{b}$<br>2592 | 53.8 ± 0.1 $^{b}$<br>2374 | 13.0 ± 0.1 $^{b}$<br>2374 |
| 57 wk | 66.4 ± 0.1 $^{a}$<br>2664 | 57.5 ± 0.10 $^{a}$<br>2076 | 13.3 ± 0.1 $^{a}$<br>2076 |

[1] Weight Loss (%) = [(egg set weight – egg weight at transfer)/egg set weight]* 100.

[2] Number of eggs.

[a-c] Means within the same column with different superscripts differ significantly ($P<0.05$).

**TABLE 2-4. Percent fertility, hatchability, hatch of fertile, early, mid and late embryonic mortality and culled chicks from eggs produced by a breeder flock at three ages**

| Breeder Flock Age | n[1] | Fertility[2] (%) | Hatchability[3] (%) | Hatch of Fertile[4] (%) | Early Dead[5] (%) | Mid Dead[6] (%) | Late Dead[7] (%) | Culls[8] (%) |
|---|---|---|---|---|---|---|---|---|
| 28 wk | 125 | 93.1 $^{b}$ | 78.8 $^{b}$ | 84.6 $^{b}$ | 5.9 $^{a}$ | 0.9 $^{b}$ | 4.9 $^{b}$ | 3.3 $^{a}$ |
| 43 wk | 144 | 96.4 $^{a}$ | 83.7 $^{a}$ | 86.8 $^{a}$ | 4.2 $^{b}$ | 1.6 $^{b}$ | 4.9 $^{b}$ | 2.1 $^{b}$ |
| 57 wk | 148 | 84.0 $^{c}$ | 63.6 $^{c}$ | 75.6 $^{c}$ | 7.0 $^{a}$ | 2.4 $^{a}$ | 10.7 $^{a}$ | 3.3 $^{a}$ |
| Pooled SEM | | 0.6 | 1.0 | 0.9 | 0.5 | 0.3 | 0.6 | 0.4 |

[1] Number of experimental units; each experimental unit = 18 eggs.

[2] Fertility (%) = (number of fertile eggs/ number of eggs set) * 100.

[3] Hatchability (%) = (total number of chicks hatched/number of eggs set) * 100.

[4] Hatch of Fertile (%) = (total number of chicks hatched/number of fertile eggs set) * 100.

[5] Early Dead (%) = (number of embryos that died between 1 to 7 d of incubation/number of eggs set) * 100.

[6] Mid Dead (%) = (number of embryos that died between 8 to 14 d of incubation /number of eggs set) * 100.

[7] Late Dead (%) = (number of embryos that died between 15 to 21 d of incubation /number of eggs set) * 100.

[8] Culls (%) = (number of chicks culled at hatching/number of eggs set) * 100.

[a-c] Means within the same column with different superscripts differ significantly ($P<0.05$).

**TABLE 2-5. Effect of breeder flock age, probiotic treatment, and the interaction on average weekly broiler body weights**

|  | n[1] | Chick Weight (g) | Wk 1 (g) | Wk 2 (g) | Wk 3 (g) | Wk 4 (g) | Wk 5 (g) | Wk 6 (g) |
|---|---|---|---|---|---|---|---|---|
| Breeder Flock Age |  |  |  |  |  |  |  |  |
| 28 wk | 480 | 38.9 ± 0.1 $^{c}$ | 93.4 ± 1.5 $^{b}$ | 214.2 ± 6.0 $^{b}$ | 408.6 ± 7.7 $^{c}$ | 722.8 ± 12.4 $^{b}$ | 1160.8 ± 16.9 $^{b}$ | 1725.47 ± 20.6 $^{b}$ |
| 43 wk | 480 | 41.5 ± 0.1 $^{b}$ | 116.6 ± 1.2 $^{a}$ | 281.5 ± 3.2 $^{a}$ | 571.1 ± 6.1 $^{b}$ | 1008.6 ± 9.8 $^{a}$ | 1534.7 ± 13.4 $^{a}$ | 2112.29 ± 16.4 $^{a}$ |
| 57 wk | 480 | 43.4 ± 0.1 $^{a}$ | 119.7 ± 1.6 $^{a}$ | 291.7 ± 4.2 $^{a}$ | 612.9 ± 8.1 $^{a}$ | 1036.2 ± 12.9 $^{a}$ | 1540.5 ± 17.7 $^{a}$ | 2099.91 ± 21.7 $^{a}$ |
| Probiotic Treatment [2] |  |  |  |  |  |  |  |  |
| CON | 360 | 41.1 ± 0.1 $^{c}$ | 109.0 ± 1.4 | 260.0 ± 3.7 | 530.2 ± 7.1 | 929.1 ± 11.4 | 1419.2 ± 15.6 | 1961.9 ± 19.0 |
| IBW | 360 | 41.3 ± 0.1 $^{bc}$ | 110.3 ± 1.3 | 263.8 ± 3.5 | 530.1 ± 6.8 | 916.2 ± 10.9 | 1406.4 ± 15.0 | 1974.0 ± 18.4 |
| IBS | 360 | 41.4 ± 0.1 $^{ab}$ | 111.1 ± 1.4 | 265.8 ± 3.7 | 534.7 ± 7.2 | 929.2 ± 11.4 | 1422.1 ± 15.7 | 1999.3 ± 19.3 |
| PAF | 360 | 41.2 ± 0.1 $^{c}$ | 109.2 ± 1.4 | 260.3 ± 3.7 | 528.5 ± 7.1 | 915.5 ± 11.3 | 4100.1 ± 15.6 | 1981.7 ± 19.0 |
| Breeder Flock Age*Probiotic Treatment |  |  |  |  |  |  |  |  |
| 28 wk * CON | 120 | 38.3 ± 0.1 $^{e}$ | 92.3 ± 2.6 | 208.0 ± 6.9 | 402.5 ± 13.3 | 732.8 ± 21.3 | 1171.2 ± 29.1 | 1723.9 ± 35.5 |
| 28 wk * IBW | 120 | 39.0 ± 0.1 $^{c}$ | 93.0 ± 2.5 | 215.5 ± 6.6 | 412.4 ± 12.7 | 704.9 ± 20.6 | 1147.3 ± 28.1 | 1704.9 ± 34.6 |
| 28 wk * IBS | 120 | 39.4 ± 0.1 $^{d}$ | 97.4 ± 2.4 | 224.9 ± 6.3 | 420.3 ± 12.3 | 739.5 ± 19.7 | 1198.8 ± 26.8 | 1774.8 ± 32.7 |
| 28 wk * PAF | 120 | 38.8 ± 0.1 $^{cd}$ | 91.0 ± 2.5 | 208.6 ± 6.6 | 399.3 ± 12.9 | 713.9 ± 20.5 | 1126.0 ± 28.1 | 1698.2 ± 34.2 |
| 43 wk * CON | 120 | 41.5 ± 0.2 $^{b}$ | 115.0 ± 2.6 | 278.8 ± 7.0 | 564.3 ± 13.5 | 998.8 ± 21.4 | 1518.5 ± 29.2 | 2084.6 ± 35.9 |
| 43 wk * IBW | 120 | 41.5 ± 0.1 $^{b}$ | 116.9 ± 2.3 | 275.8 ± 6.1 | 558.6 ± 11.7 | 987.4 ± 18.8 | 1514.3 ± 25.8 | 2106.1 ± 31.4 |
| 43 wk * IBS | 120 | 41.5 ± 0.1 $^{b}$ | 116.2 ± 2.3 | 281.0 ± 6.0 | 569.7 ± 11.8 | 1010.0 ± 18.9 | 1533.8 ± 26.1 | 2104.9 ± 32.0 |
| 43 wk * PAF | 120 | 41.4 ± 0.1 $^{b}$ | 118.1 ± 2.3 | 290.6 ± 6.0 | 591.8 ± 11.7 | 1038.1 ± 18.5 | 1572.0 ± 25.7 | 2153.7 ± 31.9 |
| 57 wk * CON | 120 | 43.5 ± 0.1 $^{a}$ | 119.8 ± 2.5 | 293.3 ± 6.6 | 623.9 ± 12.9 | 1055.7 ± 20.5 | 1567.9 ± 28.3 | 2077.1 ± 34.5 |
| 57 wk * IBW | 120 | 43.5 ± 0.1 $^{a}$ | 120.8 ± 2.5 | 300.1 ± 6.6 | 619.1 ± 12.8 | 1056.3 ± 20.3 | 1557.8 ± 27.9 | 2111.1 ± 34.3 |
| 57 wk * IBS | 120 | 43.4 ± 0.2 $^{a}$ | 119.5 ± 2.8 | 291.4 ± 7.5 | 614.2 ± 14.5 | 1038.1 ± 22.9 | 1533.7 ± 31.5 | 2118.4 ± 38.9 |
| 57 wk * PAF | 120 | 43.4 ± 0.2 $^{a}$ | 118.6 ± 2.8 | 281.9 ± 7.4 | 594.2 ± 14.4 | 994.7 ± 22.8 | 1502.4 ± 31.5 | 2093.1 ± 38.4 |

$^{a\text{-}d}$ Means within the same column with different superscripts differ significantly ($P<0.05$).

[1] Number of broilers.

[2] CON = Control, IBW = Interbac® Water, IBS = Interbac® Spray, PAF = Pro-Avi® Feed.

TABLE 2-6. Effect of breeder flock age and probiotic treatment on average weekly broiler body weight gains

| | $n^1$ | Wk 1 (g) | Wk 2 (g) | Wk 3 (g) | Wk 4 (g) | Wk 5 (g) | Wk 6 (g) | Overall $^3$ (g) |
|---|---|---|---|---|---|---|---|---|
| Breeder Flock Age | | | | | | | | |
| 28 wk | 480 | $52.7 \pm 1.1^{b}$ | $118.9 \pm 2.4^{c}$ | $197.9 \pm 4.1^{c}$ | $305.5 \pm 7.7^{b}$ | $433.5 \pm 7.4^{b}$ | $556.6 \pm 7.6^{b}$ | $1671.6 \pm 17.0^{b}$ |
| 43 wk | 480 | $75.3 \pm 1.2^{a}$ | $165.3 \pm 2.5^{b}$ | $289.3 \pm 4.3^{b}$ | $438.5 \pm 7.9^{a}$ | $527.4 \pm 7.7^{a}$ | $579.2 \pm 7.9^{a}$ | $2069.2 \pm 17.0^{a}$ |
| 57 wk | 480 | $78.1 \pm 1.2^{a}$ | $173.8 \pm 2.6^{a}$ | $320.3 \pm 4.4^{a}$ | $431.6 \pm 8.2^{a}$ | $515.8 \pm 8.0^{a}$ | $572.0 \pm 8.2^{ab}$ | $2088.7 \pm 17.0^{a}$ |
| Probiotic Treatment $^4$ | | | | | | | | |
| CON | 360 | 67.9 ± 1.4 | 150.9 ± 2.9 | 270.4 ± 5.0 | 398.1 ± 9.2 | 488.5 ± 8.9 | $541.5 \pm 9.1^{b}$ | 1926.1 ± 18.6 |
| IBW | 360 | 69.1 ± 1.3 | 153.8 ± 2.9 | 269.4 ± 4.7 | 387.1 ± 8.9 | 498.7 ± 8.9 | $575.3 \pm 8.8^{a}$ | 1941.0 ± 18.6 |
| IBS | 360 | 69.8 ± 1.4 | 154.9 ± 2.9 | 268.7 ± 5.0 | 395.9 ± 9.3 | 494.6 ± 9.0 | $578.8 \pm 9.2^{a}$ | 1962.8 ± 18.6 |
| PAF | 360 | 68.1 ± 1.4 | 151.1 ± 2.8 | 268.1 ± 5.0 | 386.3 ± 9.2 | 487.1 ± 8.6 | $581.6 \pm 9.1^{a}$ | 1942.8 ± 18.6 |

$^{a\text{-}c}$ Means within the same column with different superscripts differ significantly ($P<0.05$).

[2] Number of broilers.

[3] Overall gain = market body weight – chick weight.

[4] CON = Control, IBW = Interbac® Water, IBS = Interbac® Spray, PAF = Pro-Avi® Feed.

TABLE 2-7. Effect of breeder flock age and probiotic treatment on average weekly broiler feed conversion ratios (FCR)

|  | Wk 1 (g feed/g gain) | Wk 2 (g feed/g gain) | Wk 3 (g feed/g gain) | Wk 4 (g feed/g gain) | Wk 5 (g feed/g gain) | Wk 6 (g feed/g gain) | Overall (g feed/g gain)$^{2}$ |
|---|---|---|---|---|---|---|---|
| Breeder Flock Age |  |  |  |  |  |  |  |
| 28 wk | 1.59 ± 0.03 $^{a}$ | 1.55 ± 0.02 $^{b}$ | 1.55 ± 0.03 $^{a}$ | 1.74 ± 0.03 $^{a}$ | 1.83 ± 0.04 | 1.89 ± 0.27 | 1.73 ± 0.01 $^{b}$ |
| 43 wk | 1.31 ± 0.03 $^{b}$ | 1.73 ± 0.03 $^{a}$ | 1.54 ± 0.03 $^{a}$ | 1.63 ± 0.03 $^{b}$ | 1.82 ± 0.04 | 2.00 ± 0.28 | 1.76 ± 0.01 $^{ab}$ |
| 57 wk | 1.38 ± 0.03 $^{b}$ | 1.50 ± 0.03 $^{b}$ | 1.48 ± 0.03 $^{a}$ | 1.72 ± 0.03 $^{ab}$ | 1.83 ± 0.04 | 2.55 ± 0.29 | 1.78 ± 0.01 $^{a}$ |
| Probiotic Treatment $^{3}$ |  |  |  |  |  |  |  |
| CON | 1.44 ± 0.03 | 1.59 ± 0.03 | 1.52 ± 0.03 | 1.67 ± 0.04 | 1.84 ± 0.05 | 2.10 ± 0.32 | 1.77 ± 0.01 |
| IBW | 1.40 ± 0.03 | 1.61 ± 0.03 | 1.52 ± 0.03 | 1.71 ± 0.04 | 1.76 ± 0.05 | 2.59 ± 0.32 | 1.74 ± 0.01 |
| IBS | 1.39 ± 0.03 | 1.58 ± 0.03 | 1.53 ± 0.03 | 1.69 ± 0.04 | 1.85 ± 0.05 | 1.95 ± 0.32 | 1.75 ± 0.01 |
| PAF | 1.47 ± 0.03 | 1.60 ± 0.03 | 1.53 ± 0.03 | 1.72 ± 0.04 | 1.87 ± 0.04 | 1.94 ± 0.31 | 1.76 ± 0.01 |

$^{a\text{-}b}$ Means in the same column with different superscripts differ significantly ($P<0.05$).

[2] Overall feed conversion = total feed consumed per bird over 6 wk period/total weight gain per bird over 6 wk period.

[3] CON = Control, IBW = Interbac® Water, IBS = Interbac® Spray, PAF = Pro-Avi® Feed.

**TABLE 2-8. Effect of breeder flock age and probiotic treatment on weekly cumulative mortality of broilers**

| | Wk 1 (%) | Wk 2 (%) | Wk 3 (%) | Wk 4 (%) | Wk 5 (%) | Wk 6 (%) |
|---|---|---|---|---|---|---|
| Breeder Flock Age | | | | | | |
| 28 wk | 0.4 ± 0.2 $^{b}$ | 1.1 ± 0.3 $^{b}$ | 1.9 ± 0.4 | 2.7 ± 0.5 | 3.4 ± 0.6 | 3.6 ± 0.6 |
| 43 wk | 0.3 ± 0.2 $^{b}$ | 1.0 ± 0.3 $^{b}$ | 1.8 ± 0.4 | 2.5 ± 0.6 | 3.7 ± 0.6 | 4.6 ± 0.7 |
| 57 wk | 1.3 ± 0.2 $^{a}$ | 2.1 ± 0.3 $^{a}$ | 2.7 ± 0.4 | 3.7 ± 0.6 | 4.4 ± 0.7 | 5.1 ± 0.7 |
| Probiotic Treatment $^{2}$ | | | | | | |
| CON | 0.5 ± 0.2 | 1.4 ± 0.4 | 2.3 ± 0.5 | 3.0 ± 0.6 | 3.6 ± 0.7 | 4.1 ± 0.8 $^{ab}$ |
| IBW | 0.7 ± 0.2 | 1.4 ± 0.4 | 2.3 ± 0.5 | 3.9 ± 0.6 | 4.8 ± 0.7 | 5.3 ± 0.8 $^{a}$ |
| IBS | 0.9 ± 0.2 | 1.7 ± 0.4 | 2.6 ± 0.5 | 3.3 ± 0.6 | 4.6 ± 0.7 | 5.4 ± 0.8 $^{a}$ |
| PAF | 0.6 ± 0.2 | 1.1 ± 0.4 | 1.4 ± 0.5 | 1.7 ± 0.6 | 2.4 ± 0.7 | 2.9 ± 0.7 $^{b}$ |

$^{a\text{-}b}$ Means in the same column with different superscripts differ significantly ($P<0.05$).

$^{2}$ CON = Control, IBW = Interbac® Water, IBS = Interbac® Spray, PAF = Pro-Avi® Feed.

# REFERENCES

Annett, C. B., J. R. Viste, M. Chirino-Trejo, H. L. Classen, D. M. Middelton, and E. Simko, 2002. Necrotic enteritis: effect of barley, wheat and corn diets on proliferation of *Clostridium perfringens* type A. Avian Pathol. 31:599-602.

Ar, A., C. V. Paganelli, R. B. Reeves, D. G. Greene, and H. Rahn, 1974. The avian egg: water vapor conductance, shell thickness, and functional pore area. Condor 76:153-158.

Baba, E., S. Nagaishi, T. Fukata, and A. Arakawa. 1991. The role of intestinal microflora on the prevention of Salmonella colonization in gnotobiotic chickens. Poult. Sci. 70:1902-1907.

Barnes, E. M., 1972. The avian intestinal flora with particular reference to the possible ecological significance of the cecal anaerobic bacteria. Am. J. Clin. Nutr. 25:1475-1479.

Barnes, E. M., 1979. The intestinal microflora of poultry and game birds during life and after storage. J. Appl. Bacteriol. 46:407-419.

Bilgili, S. F., and E. T. Moran Jr., 1990. Influence of whey and probiotic-supplemented withdrawal feed on the retention of *Salmonella* intubated into market age broilers. Poult. Sci. 69:1670-1674.

Blankenship, L. C., J. S. Bailey, N. A. Cox, N. J. Stern, R. Brewer, and O. Williams, 1993. Two-step mucosal competitive exclusion flora treatment to diminish Salmonellae in commercial broiler chickens. Poult. Sci. 72:1667-1672.

Canadian Council on Animal Care, 1993. A guide to the care and use of experimental animals. Vol. 1, 2nd ed. E. D. Olfert, B. M. Cross, and A. A. McWilliams, eds. Canadian Council on Animal Care (CCAC), Ottawa, ON.

CFIA, 2001. Veterinary biologics section. http://www.inspection.gc.ca/english/anima/vetbio/vbpve.shtml. Accessed May 13, 2004.

Chambers JR, and X. Lu, 2002. Probiotics and maternal vaccination for *Salmonella* control in broiler chickens. J. Appl. Poult. Res.11:320-327.

Chateau, N., I. Castellanos, and A. M. Deschamps, 1993. Distribution of pathogen inhibition in the *Lactobacillus* isolates of a commercial probiotic consortium. J. Appl. Bacteriol. 74: 36-40.

Coates, M. E., and R. Fuller, 1977. The gnotobiotic animal in the study of gut microbiology. Pages 311-346 in Microbial Ecology of the Gut. R. T. J. Clarke and T. Bauchop (ed.). Academic Press, London, UK.

Cox, N. A., J. S. Bailey, and N. J. Stern, 2001. Effectiveness of an undefined mucosal competitive exclusion treatment to control *Salmonella* in turkeys during brooding. J. Appl. Poult. Res. 10:319-322.

Culter, G. C., 2002. Diseases of the chicken. Pages 473-542 in Commercial Chicken Meat and Egg Production. 5th Edition. D. D. Bell, W. D. Weaver Jr, (ed.), Kluwer Academic Publishers, Norwell, Massachusetts.

Estrada, A., D. C. Wilkie, and M. Drew, 2001. Administration of *Bifidobacterium bifidum* to chicken broilers reduces the number of carcass condemnations for cellulitis at the abattoir. J. Appl. Poult. Res. 10:329-334.

Fukata, T., Y. Hadate, E. Baba, and A. Arakawa, 1991. Influence of bacteria on *Clostridium perfringens* infections in young chicks. Avian Dis. 35:224-227.

Fuller, R., 1977. The importance of lactobacilli in maintaining normal microbial balance in the crop. Br. Poult. Sci. 18:85-94.

Guan, L., December 2003. personal communication. 2-50 Agriculture-Forestry Centre, Department of Agriculture, Food, and Nutritional Science, University of Alberta, Edmonton, AB, Canada, T6G 2P5.

Guan, L. L., K. E. Hagen, T. L. Grayson, G. W. Tannock, D. R. Korver, G. M. Fasenko, and G. E. Allison, 2004. Detection of *Lactobacillus acidophilus* species in the gut of chickens. Poster Presentation N147, 104th General Meeting of the American Society for Microbiology, New Orleans, LA.

Hays, F. A., and E. W. Spear, 1952. Relation of age of parents to mortality and sex ratio of chicks at eight weeks. Poult. Sci. 31:792-795.

Hearn, P. J., 1986. Making use of small hatching eggs in an integrated broiler company. Br. Poult. Sci. 27:498.

Hofacre, C. L., T. Beacorn, S. Collett, and G. Mathis, 2003. Using competitive exclusion mannan-oligosaccharides and other intestinal products to control necrotic enteritis. J. Appl. Poult. Res. 12:60-64.

Howe, K., A. H. Linton, and A. D. Osborne, 1976. The effect of tetracycline on the coliform gut flora of broiler chickens with special reference to antibiotic resistance and o-serotypes of *Escherichia coli*. J. Appl. Bacteriol. 41:453-464.

Huang, M. K., Y. J. Choi, R. Houde, J. W. Lee, B. Lee, and X. Zhao, 2004. Effects of lactobacilli and an acidophilic fungus on the production performance and immune responses in broiler chickens. Poult. Sci. 83:788-795.

Hume, M. E., L. F. Kubena, T. S. Edrington, C. J. Donskey, R. W. Moore, S. C. Ricke and D. J. Nisbet, 2003. Poultry digestive microflora biodiversity as indicated by denaturing gradient gel electrophoresis. Poult. Sci. 82:1100-1107.

Jin L.Z., Y. W. Ho, M. A. Ali, N. Abdullah, and S. Jalaludin, 1998. Growth performance, intestinal microbial populations, and serum cholesterol of broilers fed diets containing *Lactobacillus* cultures. Poult. Sci. 77:1259-1265.

Jones, F.T., and K. E. Richardson, 2004. *Salmonella* in commercially manufactured feeds. Poult. Sci. 83:384-391.

Kubena, L. F., R. H. Bailey, J. A. Byrd, C. R. Young, D. E. Corrier, L. H. Stanker, and G. E. Rottinghaus, 2001. Cecal volatile fatty acids and broiler chick susceptibility to *Salmonella typhimurium* colonization as affected by aflatoxins and T-2 toxin. Poult. Sci. 80:411-417.

LaRagione, R. M., G. Casula, S. M. Cutting, and M. J. Woodward, 2001. *Bacillus subtilis* spores competitively exclude *Escherichia coli* O78:K80 in poultry. Vet. Microbiol. 79:133-142.

Lerner, I. M., and C. A. Gunns, 1952. Egg size and reproductive fitness. Poult. Sci. 31:537-544.

McClung, M. R., and R. M. Smith, 1949. Relationship between egg weight and growth rate of market chickens. Poult. Sci. 28:774.

McNaughton, J. L., J. W. Deaton, F. N. Reece, and R. L. Haynes, 1978. Effect of age of parents and hatching egg weight on broiler chick mortality. Poult. Sci. 57:38-44.

Mead, G. C., 2000. Prospects for competitive exclusion treatment to control salmonellas and other foodborne pathogens in poultry. Vet. J. 159:111-123.

Mead, G. C., and B. W. Adams, 1975. Some observations on the cecal microflora of the chick during the first two weeks of life. Br. Poult. Sci. 16:169-176.

Merritt, E. S., and R. S. Gowe, 1965. Post embryonic growth in relation to egg weight. Poult. Sci. 44:477-480.

Mohan, B., R. Kadirvel, A. Natarajan, and M. Bhaskaran, 1996. Effect of probiotic supplementation on growth, nitrogen utilization and serum cholesterol in broilers. Br. Poult. Sci. 37:395-401.

Morris, R. H., D. F. Hessels, and R. J. Bishop, 1968. The relationship between hatching egg weight and subsequent performance of broiler chickens. Br. Poult. Sci. 9:305-315.

Nisbet, D. J., D. E. Corrier, and J. R. DeLoach, 1993. Effect of mixed cecal microflora maintained in continuous culture and of dietary lactose on *Salmonella typhimurium* colonization in broiler chicks. Avian Dis. 37:528-535.

Nurmi E., and M. Rantala, 1973. New aspects in *Salmonella* infections in broiler production. Nature. 241:210-211.

O'Neil, J. B., 1950. Relationship of chick size to egg size and its effect upon growth and mortality. Poult. Sci. 29:774(Abstract).

O'Neil, J. B., 1955. Percentage size of chick at hatching and its relationship to growth and mortality. Poult. Sci. 34:761-764.

Palmu, L., and I. Camelin, 1997. The use of competitive exclusion in broilers to reduce the level of *Salmonella* contamination on the farm and at the processing plant. Poult. Sci. 76:1501-1505.

Pascual, M., M. Hugas, J. I. Badiola, J. M. Monfort, and M. Garriga, 1999. *Lactobacillus salivarius* CTC2197 prevents *Salmonella enteritidis* colonization in chickens. Appl. Environ. Microbiol. 65:4981-4986.

Pinchasov Y., and Y. Noy, 1993. Comparison of post-hatch holding time and subsequent early performance of broiler chicks and turkey poults. Br. Poult. Sci. 34:111-120.

Proudfoot, F. G., and H. W. Hulan, 1981. The influence of hatching egg size on the subsequent performance of broiler chickens. Poult. Sci. 60:2167-2170.

Qureshi, M. A., and G. B. Havenstein, 1994. A comparison of the immune performance of a 1991 commercial broiler with a 1957 randombred strain when fed "typical" 1957 and 1991 broiler diets. Poult. Sci. 73:1805-1812.

Riddell, C., and X. M. Kong, 1992. The influence of diet on necrotic enteritis in broiler chickens. Avian Dis. 36:499-503.

SAS Institute, 1999. SAS user's guide version 8. SAS Institute Inc., Cary, NC.

Schleifer, J. H., 1985. A review of the efficacy and mechanism of competitive exclusion for the control of *Salmonella* in poultry. Worlds Poult. Sci. 41:72-83.

Sklan, D., S. Heifetz, and O. Halevy, 2003. Heavier chicks at hatch improves marketing body weight by enhancing skeletal muscle growth. Poult. Sci. 82:1778-1786.

Sinclair, R. W., F. E. Robinson, and R. T. Hardin, 1990. The effects of parent age and posthatch treatment on broiler performance. Poult. Sci. 69:526-534.

Smith, H. W., 1965. The development of the flora of the alimentary tract in young animals. J. Pathol. Bacteriol. 90:495-513.

Tortuero, F., 1973. Influence of the implantation of *Lactobacillus acidophilus* in chicks on the growth, feed conversion, malabsorption of fat syndrome and intestinal flora. Poult. Sci. 52:197-203.

Watkins, B. A., and F. H. Kratzer, 1983. Effect of oral dosing of *Lactobacillus* strains on gut colonization and liver biotin in broiler chicks. Poult. Sci. 62:2088-2094.

Watkins, B. A., and F. H. Kratzer, 1984. Drinking water treatment with a commercial preparation of a concentrated *Lactobacillus* culture for broiler chickens. Poult. Sci. 63:1671-1673.

Wyatt, C. L., W. D. Weaver Jr., and W. L. Beane, 1985. Influence of egg size, eggshell quality, and post-hatch holding time on broiler performance. Poult. Sci. 64:2049-2055.

Yeo, J., and K. I. Kim, 1997. Effect of feeding diets containing an antibiotic, a probiotic, or yucca extract on growth and intestinal urease activity in broiler chicks. Poult. Sci. 76:381-385.

Zulkifli, I., N. Abdullah, N. M. Azrin, and Y. W. Ho, 2000. Growth performance and immune response of two commercial broiler strains fed diets containing *Lactobacillus* cultures and oxytetracycline under heat stress conditions. Br. Poult. Sci. 41:593-597.

# 3. GENERAL CONCLUSIONS

There are many conflicting reports on the efficacy of probiotic bacteria in improving broiler growth and production efficiency, and there are many reasons why the results of trials administering probiotics to broiler chickens differ so drastically. Some experiments use commercial products (Blankenship *et al.*, 1993; Chambers and Lu, 2002), whereas others use pure, and highly potent laboratory cultures (Jin *et al.*, 1998; Estrada *et al.*, 2001). There is also the issue of differences observed in the efficacy of undefined or complex cultures compared to simple cultures or single organisms (Humbert *et al.*, 1989; Baba *et al.*, 1991).

The results of the present experiment do not allow a judgment to be made on the efficacy of probiotic cultures in general, but only on the efficacy of the specific products and microorganisms examined under the specific diet and rearing conditions used. In this experiment, it was found that the probiotic products investigated provided no improvements in broiler growth characteristics at any breeder flock age. For a more comprehensive picture of any other effects of these probiotics *in vivo*, studies would need to be conducted which examine the ability of the strains included in the probiotics to adhere to the GIT epithelium. A comparison of the numbers of pathogenic, or undesirable organisms able to colonize the GIT of broilers in each treatment group would also be of value in determining the true effectiveness of these products in altering the GIT microflora.

In general, more information is needed with regard to the composition of the chicken GIT commensal microflora, and its interactions with the GIT epithelial cells in vivo. Once this information is available, more focused efforts can be applied to the development of probiotic products that will improve broiler growth and production efficiency.

# REFERENCES

Baba, E., S. Nagaishi, T. Fukata, and A. Arakawa. 1991. The role of intestinal microflora on the prevention of Salmonella colonization in gnotobiotic chickens. Poult. Sci. 70:1902-1907.

Blankenship, L. C., J. S. Bailey, N. A. Cox, N. J. Stern, R. Brewer, and O. Williams, 1993. Two-step mucosal competitive exclusion flora treatment to diminish salmonellae in commercial broiler chickens. Poult. Sci. 72:1667-1672.

Chambers, J. R., and X. Lu. 2002. Probiotics and maternal vaccination for *Salmonella* control in broiler chickens. J. Appl. Poult. Res. 11:320-327.

Estrada, A., D. C. Wilkie, and M. Drew, 2001. Administration of *Bifidobacterium bifidum* to chicken broilers reduces the number of carcass condemnations for cellulitis at the abattoir. J. Appl. Poult. Res. 10:329-334.

Humbert, F., G. Salvat, M. Morin, P. Colin, C. Lahellec, and G. Bennejean, 1989. La colonization spontanee de la muqueuse caecale du poulet exempt d'organismes pathogens specifies et l'implantation provoquee d'une flore de barriere chez le poulet conventionnel: etude au microscope electronique a baylage. Avian Pathol. 18:577-589.

Jin, L. Z., Y. W. Ho, N. Abdullah, and S. Jalaludin, 1998. Growth performance, intestinal microbial populations, and serum cholesterol of broilers fed diets containing *Lactobacillus* cultures. Poult. Sci. 77:1259-1265.

# APPENDIX: INVESTIGATING THE MICROBIOLOGICAL CONTENT OF TWO COMMERCIALLY AVAILABLE PROBIOTIC SUPPLEMENTS FOR BROILER CHICKENS

## INTRODUCTION

Research has shown that the composition of probiotic products is one of the most important factors in determining their efficacy in delivering the advertised product benefits (Haddadin *et al.*, 1996; Cox *et al.*, 2001). Incidences of commercial probiotic products for human consumption either containing microorganisms not listed on the label, not containing microorganisms listed on the label, or containing the appropriate microorganisms, but not in the quantities guaranteed by the manufacturer have been reported (Hamilton-Miller *et al.*, 1999; Temmerman *et al.*, 2003). Any of these errors can contribute to a lowered efficacy of the probiotic product.

One study conducted in the United Kingdom (Hamilton-Miller *et al.*, 1999) examined the contents of 21 commercial probiotic supplements for humans, and discovered some very disturbing trends. Many of the products examined failed to meet the claims of the manufacturers with regard to both the bacterial species present (qualitatively) and the numbers of viable organisms contained (quantitatively). In some cases, the misrepresentations of the product involved the use of outdated taxonomy, listing organisms such as *Lactobacillus bifidus*, reclassified as *Bifidobacterium bifidum* over two decades ago (Sgorbati *et al.,* 1995), and mislabeling *Enterococcus* species as *Streptococcus* (reclassified in 1984). Of the 20 products tested, 12 were missing one or more of the bacterial species claimed by the manufacturer or contained species that had been misidentified (Hamilton-Miller *et al*., 1999). Five of the products were found to be contaminated with either *E. faecium*, or a *Pediococcus* species not listed on the label. Quantitatively, only seven of the 21 products evaluated met or exceeded the numbers of viable bacteria claimed by the manufacturer. Nine of the probiotic supplements contained numbers of viable bacteria 90% lower than guaranteed by the manufacturer, and one product was found to contain no viable bacteria at all. Of the 21 products tested, only seven were found to be satisfactory, both qualitatively and quantitatively.

Another study investigated the qualitative and quantitative composition of 30 commercial probiotic supplements for humans, and 25 probiotic dairy products (Temmerman *et al*., 2003). The results determined that 47% of supplements, and 40% of the dairy products had been mislabeled in some way. Four of the 30 supplements contained additional microorganisms not listed by the manufacturer, and six products contained *E. faecium*, the inclusion of which is still controversial in commercial probiotics. Twenty-two of the 30 supplements claimed to include *L. acidophilus*, but the presence of this species was only detected in two of the supplements. Perhaps the most astounding finding was that 37% of the supplements tested were not found to contain any viable bacteria.

Based on the results of these two studies, the inaccuracies in the quantitative and qualitative composition of commercial probiotic products appears to be a common problem in products marketed for human consumption. Research in this thesis showed that two probiotic products approved for use in poultry had no effect on broiler weight gain, feed conversion, or mortality when compared to a control group. This was in contrast to previous literature indicating that probiotics can result in improved weight gains (Nurmi and Rantala, 1973; Tortuero, 1973), and feed conversion (Jin et al., 1998; Zulkifli *et al.*, 2000) in broiler chickens. Based on the above, the decision was made to investigate the qualitative and quantitative composition of the poultry probiotic products used in the research presented in this thesis.

The objectives of this research were to: 1) use selective plating methods to analyze the microbiological contents of Interbac®[7] and Pro-Avi®[1] (the only two probiotics approved for use in poultry in Canada), 2) determine the quantities of microorganisms present (colony forming units (CFU)/g), and 3) use various molecular biology techniques to identify the viable bacterial species present. Performing a qualitative and quantitative analysis of these probiotic products can verify the actual dose administered to the broiler chickens in the research outlined in this thesis. Identification of the viable bacterial species in the products will also allow for a better understanding of the potential actions of these microorganisms in the gastrointestinal tract (GIT) (ie: ability to adhere to the GIT walls).

## MATERIALS AND METHODS

### Probiotic Products

The Pro-Avi® examined in the present study was of the same lot as that which was used in the broiler research trials. It was not possible to obtain Interbac® of the same lot number used in the broiler research trials. Both products were obtained directly from the manufacturer[8] and contained freeze-dried bacteria. Prior to microbiological analysis, each product was stored in its freeze-dried powdered form, in a sealed container at 4°C in a dark room to ensure the best possible preservation of the microorganisms in the products.

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[7] Intervet Canada, Inc., 250 Water Street, Whitby, ON, Canada, L1N 9T5.

[8] Intervet Canada Inc., Whitby, ON, Canada, L1N 9T5.

The product labeling, as well as the compendium of veterinary products (Canadian Animal Health Institute, 2003) states that Interbac® contains *L. acidophilus*, *L. bifidus*, and *S. faecalis* (which has been reclassified as *E. faecalis* since 1984) and contains a total of 1x$10^7$ CFU of lactic acid bacteria per 5000 dose vial. Pro-Avi® product labeling guarantees a total of 1x$10^7$ CFU of lactic acid bacteria (LAB) per gram of product, (of *L. acidophilus*, *S. faecalis* (reclassified as *E. faecalis* in 1984), and bifidobacteria).

## Microbiological Analysis of Probiotic Products

Each product was opened, and serial dilutions ranging from $10^{-1}$ to $10^{-9}$ were immediately prepared. Separate sets of dilutions were conducted under anaerobic (10% $H_2$, 5% $CO_2$ and balance $N_2$) and aerobic conditions for both probiotic products. Aerobic dilutions were prepared in sterile 0.85% NaCl solution. Each of these dilutions were then used to inoculate Man Rogosa Sharp (MRS)[9] and *Lactobacillus* Selective (LBS)[10] agar plates, (incubated anaerobically), as well as MRS and M17[11] plates, (incubated aerobically). Anaerobic dilutions for both probiotic products were prepared in Brain Heart Infusion (BHI)[12] broth that had been pre-reduced by incubation under anaerobic conditions for 48 h prior to use. These dilutions were subsequently used to inoculate BHI agar plates, which were incubated anaerobically. All agar plates were incubated at 37°C for 48 h. Each type of media used was selected to culture a specific group of target organisms (Table A-1). For example, the pH of MRS medium is approximately 6.5, making it a suitable environment for the cultivation of most lactic acid bacteria, including those of interest in the present study. Conversely, LBS medium has a pH of approximately 5.5, making it a more selective environment for the isolation of lactobacilli, which survive at a lower pH. The aerobic MRS was chosen in an attempt to cultivate streptococci and enterococci, whereas the aerobic M17 was intended for the cultivation of streptococci and enterococci, as well as any other aerobes present. The pre-reduced BHI plates were included for the specific purpose of cultivating strict anaerobes such as bifidobacteria.

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[9] SBD Diagnostics, Sparks, MD, USA.
[10] BBL, Beckton Dickinson and Company, Cockeysville, MD, USA.
[11] Difco Laboratories, Detroit, MI, USA.
[12] Oxoid Ltd., Basingstoke, Hampshire, UK.

After incubation, colonies present on plates containing between 20 and 200 colonies were enumerated. These total lactic acid bacteria counts were subsequently used to calculate the number of colony forming units (CFU) per g of probiotic product for both Pro-Avi® and Interbac®. Separate calculations were performed to calculate the CFU/g for each set of plates (anaerobic BHI, MRS, and LBS, and aerobic MRS and M17).

Wherever sufficient colonies were present, three colonies with the same morphological characteristics were chosen for further study. These were identified by a letter assigned to the morphological type followed by "1", "2" and "3" to denote the colony number. Only colonies that were clearly separate from all other colonies were chosen in order to ensure a pure culture. The plates streaked from each of the original colonies were then incubated for 48 h at 37°C. Both the medium chosen and the aeration conditions used for this subsequent plating varied with the medium and aeration conditions originally used to culture the colony (Table A-1). The selection of a restreaking medium for each isolate was made based on which medium was more likely to provide more bacterial growth. For example, colonies isolated on anaerobic LBS were restreaked on anaerobic MRS since it is a less restrictive medium, which would result in higher growth.

After the incubation period had elapsed, the purity of each of the plates was evaluated. If a single plate was found to contain colonies with differing morphological characteristics, it was deemed to be impure, and each of the colony types present on the plate were re-streaked onto fresh plates (Table A-1). In order to differentiate between the two colony types, a letter was added to the end of each previously described colony designation (1A, 1B, etc.). These plates were incubated for 37°C for 48 h.

Once pure cultures were obtained from each colony, loopfuls of culture were used to inoculate fresh plates of the same medium previously used (Table A-1). This re-streaking was necessary to obtain sufficient quantities of bacterial cells for both DNA extraction and to make stock solutions of the culture. All plates were then incubated for 48 h at 37°C.

After incubation, half the cells cultured from each colony were harvested and placed in 1 ml of 50% glycerol stock solution (Table A-1), which was then frozen at –80°C. The remaining cells from each culture were placed in 1ml of sterile, purified water for DNA extraction.

An overview of the procedures described above for the microbiological analysis of the probiotic products is summarized in Figure A-1.

## Molecular Analysis of Probiotic Bacterial Strains

DNA extraction from the probiotic isolates was carried out as described by Walter *et al.*, (2000). The tubes containing the cell culture and sterile water were centrifuged for three min at 14,600 X *g* and 4°C[13] (standard centrifugation conditions unless otherwise specified). The pellet in each tube was re-suspended in one ml of TN 150 buffer (10 mM Tris-HCl (pH 8.0), 150 mM NaCl) to wash the cells and remove any extracellular debris from the samples. The samples were centrifuged, and the cells re-suspended in TN 150 buffer. The cells and TN 150 buffer solution were transferred to a sterilized beadbeater tube containing 0.3 g zirconium beads (0.1 mm diameter), and homogenized for 3 min using a beadbeater[14]. This process physically destroyed the cells, freeing the DNA into the supernatant. The tubes were allowed to cool on ice until all the zirconium beads had settled to the bottom, at which point 500 µl of supernatant was removed to a new tube. This supernatant portion contained all DNA, RNA, and proteins from the isolate.

At this point, phenol:chloroform extraction was performed. First, 500 µl of TE buffer-saturated phenol (pH 8.0) was added to each tube to remove proteins from the supernatant. The tubes were centrifuged and the aqueous layer transferred to a new tube. Addition of 500 µl of chloroform-isoamyl alcohol (24:1) was done to remove remaining phenol from the aqueous layer. The tubes were centrifuged and the aqueous layer transferred to a new tube. After addition of 50 µl of 3M Sodium Acetate, and 1 ml of cold 95% ethanol, the tubes were incubated overnight at –20°C. This ethanol precipitation procedure allowed the DNA and RNA in the isolate to be precipitated out of the solution, while other cellular components remained dissolved in the ethanol.

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[13] Thermo IEC Micromax RF Centrifuge, IEC, Inc. Needham Heights, MA, USA, 02494.
[14] Mini-beadbeater, Biospec Products Inc., Bartlesville, OK, USA, 74005.

After incubation, the tubes were centrifuged for 20 min at 14,600 X *g* and 4°C to precipitate the DNA and RNA out of the solution. The ethanol mixture was poured off, and the tubes were dried for 1 h at 37°C to remove any remaining ethanol. The pellets (containing DNA and RNA) were dissolved in 500 µl of 1 x TE buffer (10 mM Tris-HCl, 1 mM EDTA). Twenty-five µl of 2 mg/ml RNAse was added to each tube, and the tubes incubated for 1 hour at 37°C to degrade any RNA present in the sample. The phenol:chloroform extraction was then repeated to remove the RNAse and degraded RNA from the samples and the ethanol precipitation was repeated, as previously outlined, to precipitate the DNA.

The DNA pellets were dissolved in 20 µl of 1 X TE buffer, which was used for DNA storage after extraction. The 1 X TE buffer was made from a 10 X concentrated stock solution as needed (100mM Tris-Cl and 10mM EDTA) (Sambrook and Russell, 2001b). The presence of DNA in each sample was confirmed by gel electrophoresis before proceeding. In the present study, standard electrophoresis conditions were as follows: horizontal electrophoresis using either 0.7% or 2% agarose gel[15], run at a voltage not exceeding 100 V. A 1 X TBE buffer (Sambrook and Russell, 2001b) was used as the electrophoresis buffer, as well as to make all agarose gels. A 6 X concentrated buffer containing 0.25% bromophenol blue, and 30% glycerol in purified water (Sambrook and Russell, 2001b; L. Guan, personal communication) was used as a gel loading buffer. In every gel, a 1 kb ladder[16] was run alongside samples to facilitate the determination of fragment sizes, and comparisons between gels.

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[15] Ultra Pure™ Agarose, Invitrogen Canada Inc., Burlington, ON, Canada.

[16] Invitrogen Canada Inc. Burlington, ON, Canada, L7P 1A1.

After the DNA had traveled the length of the gel, as evidenced by the progress of the gel loading buffer, the applied voltage was stopped, and the gel was immersed in ethidium bromide (0.5 µg/ml) staining solution (EtBr). EtBr is a fluorescent dye, which incorporates itself between the stacked bases of the DNA (Sambrook and Russell, 2001a). The dye binds to the DNA, causing increased fluorescence, allowing even small amounts of DNA to be visualized as fluorescent bands when the gel was photographed using a 300 nm UV light source (Sambrook and Russell, 2001a). In order to verify the presence of DNA in each of the isolates, one µl of DNA extract and 1 µl of 6 X loading dye were run on the gel.

***RAPD PCR Analysis.*** The DNA samples were diluted to 1/20 with 1 X TE buffer prior to the polymerase chain reaction (PCR). Random Amplification of Polymorphic DNA (RAPD) was then performed according to the protocol of Roy *et al.* (2000). The reagents and primers used in the RAPD PCR reaction mixture are outlined in Table A-2. The time and temperature profile used for RAPD PCR in the present study is shown in Figure A-2. A negative control was included in all PCR reactions in order to ensure the purity of the reagents and that the banding patterns observed after gel electrophoresis were the sole result of the amplification of template DNA from the isolates. The control contained all reagents used in the PCR reaction mixture but the sample DNA was replaced with an equivalent volume of sterile water.

The primer OPA-02 was used for all RAPD PCR reactions in the present study (Roy *et al.*, 2000). This primer serves to amplify a complementary sequence in the template DNA that occurs randomly throughout the bacterial chromosome, but in a strain specific manner. Since the chromosomal DNA sequence varies between different bacterial strains, different bacterial strains produce different sized DNA fragments, and hence, different banding patterns after gel electrophoresis. Samples of the same bacterial strain produce identical, or nearly identical, RAPD banding patterns. In this way, RAPD PCR was used in the present study to differentiate between isolates at the strain level, and eliminate duplicate strains among those colonies originally selected for analysis.

After the RAPD PCR reaction, 15 µl of the resulting RAPD PCR products and 3 µl of 6 X gel loading buffer were run on a 2% agarose gel. Banding patterns were visualized using 300 nm UV light after the gel had been stained with EtBr.

If amplification failed, as evidenced by the lack of a RAPD PCR product, the concentration of DNA used in the reaction mixture was adjusted, and the PCR was repeated. Once RAPD profiles had been obtained for each of the isolates, isolates were grouped according to similarity of banding patterns (RAPD groups).

***PCR-ARDRA Analysis.*** Once the isolates had been grouped, a single isolate from each RAPD group was chosen as a representative. These representatives, along with several type strain DNA samples, were analyzed using Amplified Ribosomal DNA Restriction Analysis (ARDRA) to differentiate between the unknown isolates at the species level and identify the unknown isolates by comparison to type strain DNA (Guan *et al.*, 2003). All reagents used in the PCR-ARDRA reaction mixture are listed in Table A-3. All type strains for DNA extraction were obtained from American Type Culture Collection (ATCC)[17], and represented the bacterial species thought to be most likely to be those included in both probiotic products. The time and temperature profile used in the ARDRA PCR is shown in Figure 3. Two primers were used for PCR-ARDRA, Lb16A (Guan *et al.*, 2003) and 231B (Tannock *et al.*, 1999). These primers are designed specifically to amplify the 16S rRNA, and the 16S – 23S rRNA intergenic region of the bacterial chromosome.

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[17] American Tissue Culture Collection, Manassas, VA, USA, 20108.

The successful amplification of PCR-ARDRA products was confirmed with agarose gel electrophoresis in the same manner as previously described, except that five μl of PCR product and one μl of 6 X gel loading buffer were run in each lane of the gel. Upon verification of amplification, solutions were prepared containing 17 μl of PCR-ARDRA product, 2.0 μl of 10X *Hae*III enzyme buffer, and 1.0 μl of *Hae*III[18], a restriction enzyme. The restriction enzyme recognizes specific sequences in the DNA and cuts the DNA at these locations. After the DNA has been cleaved, the fragments can be separated from each other using gel electrophoresis (Madigan *et al.*, 2003).

The restriction enzyme reaction mixture was incubated for 3 h at 37°C. After incubation, 2 μl of sodium acetate and 66 μl of cold 95% ethanol were added to each tube, to precipitate DNA, as previously described. The DNA pellet was dissolved in 10 μl of 1 X TE buffer and 2 μl of 6 X loading dye was added to the enzymatically digested PCR products. The entire 12 μl was run on a 2% agarose gel, and the different banding patterns compared against the patterns generated by the type strain DNA. Since the sequences of the 16S and 23S rRNA genes are species-specific, the banding patterns generated from the restriction digest of these genes can be used to determine the species of each isolate. Where necessary, additional restriction enzyme digestions were conducted, such as cleaving the PCR-ARDRA products with *Hae*III for comparison against additional reference strains to identify *Enterococcus* species, or digestion with the restriction enzyme *Mse*I to further differentiate between some *Lactobacillus* species identified in the initial *Hae*III digestion.

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[18] New England Biolabs Inc., Beverly, MA, USA, 01915-5599.

# RESULTS & DISCUSSION

## Quantitative Microbiological Analysis

Both Interbac® and Pro-Avi® had considerably lower numbers of colonies on LBS plates than on any of the other media used (Table A-4). The aerobically incubated MRS and M17 plates incubated allowed for the cultivation of similar numbers of colonies as the anaerobically incubated MRS plates.

There are many media available for culturing bacteria, each with certain characteristics that make them more or less favorable for culturing specific groups or species of bacteria. In the present study, BHI agar was used exclusively for the cultivation of strict anaerobes, such as *Bifidobacterium* species, but the only colonies isolated on this medium were *L. johnsonii, L. salivarius* and *E. faecalis*: all facultative anaerobes. These organisms were all cultured in greater numbers on LBS and MRS agar, so in this particular case, nothing was gained from using BHI agar plates. It was also shown that *E. faecalis* and *L. johnsonii*, as well one or more unidentified species, can be cultivated on aerobically incubated MRS and M17. In general, it was found that most of the media used were able to cultivate all the species identified in the products with the exception of the bifidobacteria claimed by the manufacturer that could not be accounted for by any of the isolates from any medium. This explains the small differences in total lactic acid bacteria counts seen between the different media. The exception to this was the anaerobic LBS plates, on which notably lower numbers of CFU were cultured, compared to any other media used in the study. This occurred as a result of the low pH of the LBS, designed for the isolation of lactobacilli and the inhibition of other bacteria.

This is confirmed by the fact that only *L. johnsonii* and one unidentified species were able to grow on LBS plates.

Even without the isolation of the *L. bifidus* claimed by the manufacturer, the guarantee of $1 \times 10^7$ CFU/g was exceeded for the Pro-Avi®. When the CFU per vial was calculated for Interbac®, using the most generous estimate of CFU/g (on anaerobic MRS), the number of CFU/vial was $3.56 \times 10^{10}$, also exceeding the manufacturer's guarantee. The only medium that did not show sufficient growth to meet these guarantees was the anaerobically incubated LBS.

The number of CFU per dose when each product was administered to the broiler chickens was calculated theoretically using the number of CFU/g calculated from enumeration of colonies on anaerobic MRS medium. The resulting theoretical doses are $7.12 \times 10^6$ CFU/bird for Interbac® and $6.41 \times 10^7$ CFU/bird in Pro-Avi® (Table A-5). These theoretical doses were then compared to actual doses, calculated from the CFU obtained when feed and water samples collected during the field trials were plated on MRS and incubated anaerobically (L. Guan, personal communication). For both theoretical and actual doses, a dose was calculated as the amount of the product administered to a single bird over its lifespan when administered as specified by the manufacturer. For actual doses, this would be an approximation, since the dose received by a single bird would depend to a large extent on the amount of feed consumed. In the case of Pro-Avi® where the product was administered in the feed, the dose calculated was based on the average lifetime feed consumption of the birds included in the broiler trials conducted as part of this thesis.

When the theoretical values for CFU/dose were compared to actual CFU/dose values inconsistencies were evident. For Interbac®, when administered both as a spray and in the drinking water, the actual dose was a great deal lower than the theoretical dose. This may play a role in explaining why no significant differences in weight gains or feed conversion were observed in the broiler trials, since the actual dose per bird may be too low to provide any real benefit to the bird (Table A-5). Due to difficulties with supply, the Interbac® used in the broiler trials was of a different lot than the Interbac® used in the present microbiological analysis; the potential exists that the difference between theoretical and actual doses is the result of differences between lots. However, for a commercial product to be effective and approved by the CFIA, there must be consistency between lots, so the likelihood that the differences in calculated dosages were due to differences among lots is low.

When Pro-Avi® was examined, the actual dose was very similar to the theoretical dose. However, when a negative control feed sample (feed without any probiotic supplementation) was analyzed it was found to contain almost as many CFUs as the feed supplemented with Pro-Avi®. This indicates that the actual dose of organisms provided by the Pro-Avi® was almost negligible, and that the feed was contaminated with bacteria from other sources, as has been shown to occur in previous research (Jones and Richardson, 2004). It was also noted in the analyses of feed from the broiler trials that the morphologies of the colonies from feed with and without Pro-Avi® were quite different. However, no attempt was made to identify the species present in each of these samples (L. Guan, personal communication). It is possible that while the numbers of bacteria in the Pro-Avi® and control feeds are similar, the administration of Pro-Avi® in the feed may alter the dynamics of the bacterial communities. Further analysis of these feed samples would be necessary to identify which bacterial species are present, allowing the potential effects of these organisms on the broiler chickens to be examined. Until the bacterial species contained in both the control and Pro-Avi® feed are known, it is impossible to speculate on the effects of the organisms in the GIT, and the reasons why no improvements in broiler performance were noted in the broiler trials. It is, however, doubtful that the very low increase in the CFU found in Pro-Avi® feed provided to the chickens would have been sufficient to cause any beneficial effects within the birds.

For the host to benefit from the administration of a probiotic product, the beneficial bacteria must be present in sufficient numbers to competitively exclude the pathogenic bacteria present in the GIT (Blankenship *et al.*, 1993). It has been suggested that the minimum effective dose for broiler chickens is approximately $1 \times 10^5$ CFU/bird (Pascual *et al.*, 1999). The theoretical CFU/dose is above this minimum effective dose for both Interbac® and Pro-Avi®, so in theory, they should contain a sufficient number of bacteria to be effective.

Based on differing morphologies between colonies, a total of 13 anaerobic and four aerobic colony types were isolated from Interbac® (Table A-6). Seven anaerobic and five aerobic colony types were isolated from Pro-Avi® (Table A-6). Each of these colonies underwent further qualitative analysis.

### Qualitative Analysis and Speciation of Isolates

DNA was successfully extracted from all colonies, and used as a template in the RAPD PCR to compare the identity of the various isolates at the strain level. Because the

RAPD PCR reaction failed to amplify a product when DNA from isolates A-1, A-2, A-3, B-1, D-1, F-2, W-2, and X-1 (all from Interbac®), was diluted 1/20, the reaction was repeated using DNA diluted to 1/50 (Figures A-4 and A-5). There were no isolates from the Pro-Avi® for which the DNA concentration had to be adjusted.

The banding patterns visible when the RAPD PCR products were run on a gel were compared by estimating the size of each of the bands present in the samples by comparison to a ladder with fragments of known sizes. It was found that the isolates cultured from Interbac® under anaerobic conditions had nine distinct banding patterns while the colonies isolated from Interbac® under aerobic conditions had eight differing banding patterns (Figures A-4, A-5, A-6, A-7, A-8). Some contamination of the negative control used in the RAPD PCR was visible in one of the gels containing the PCR products of isolates cultured from Interbac® under anaerobic conditions. This contamination was evidenced by the presence of banding patterns, indicating DNA fragments, in the negative control (Figure A-7). However, the banding pattern in the contaminated control lane did not interfere with, and shared no common banding patterns with the banding patterns observed for the isolates, and was therefore ignored. This was the only incidence in which contamination of a negative control in a PCR reaction was observed to have occurred. Since no banding was observed in the negative control in all other gels, this lane is not included in the figures in this paper.

Ten distinct RAPD PCR banding patterns were obtained from colonies isolated from Pro-Avi® under anaerobic conditions, and seven RAPD PCR banding patterns were seen in isolates cultured from Pro-Avi® under aerobic conditions (Figures A-9 and A-10). After the initial RAPD PCR reaction produced products with no similar banding patterns among any of the isolates (data not shown), the phenol:chloroform extraction and ethanol precipitation of DNA were repeated for all isolates obtained from Pro-Avi®. The initial lack of similarity among banding patterns of the RAPD PCR products may have been due to impurities present in the DNA samples, which can inhibit the PCR reaction (L. Guan, personal communication). After repetition of the phenol:chloroform extraction and ethanol precipitation banding patterns were easily identified for each of the isolates, and grouped according to similarity.

Once isolates were grouped according to similar banding patterns, a total of 17 RAPD groups for each of Interbac® and Pro-Avi® were recognized. A representative of each RAPD group (Table A-7) was then further analyzed and identified at the species level using an ARDRA analysis.

DNA from each representative isolate was used in an ARDRA PCR reaction to amplify the 16S-23S rDNA region of the bacterial chromosome. After digesting the DNA of the RAPD PCR group representatives and some reference type strains with *Hae*III, the ARDRA products from the representative isolates were identified based on similarity of their banding patterns compared to reference strains.

All Interbac® isolates, with the exception of those belonging to RAPD groups 10, 16 and 17, matched the banding pattern for *L. gasseri* after digestion with *Hae*III (Figures A-11 and A-12). However, since it is well known that *L. gasseri* and *L. johnsonii* produce a common banding pattern when digested with *Hae*III (Guan *et al*., 2003), the ARDRA PCR products of representative isolates and reference strains were digested with the restriction enzyme *Mse*I, which results in different banding patterns for each of these two species. Based on the *Mse*I digestion, the isolates from RAPD groups 1 to 9 and 11 to 15 were identified as *L. johnsonii* (ATCC 33200) (Figures A-13 and A-14). A third enzyme digestion was necessary to compare representative isolates from RAPD groups 10, 16 and 17 to *E. faecalis* and *E. faecium* after digestion with *Hae*III, since these two reference strains had not been included in the initial ARDRA PCR reaction and enzyme digestion. After this third enzyme digestion, the isolates from RAPD groups 16 and 17 were identified as *E. faecalis* (Figure A-15). The isolates from RAPD group 10 were not identifiable as belonging to any of the reference strains used in the present study (Figures A-12, A-14, and A-15). As they do not appear to be of any of the species listed by the manufacturer, nor any of the reference strains thought likely to be contained in probiotic products, comparison to additional reference strains would be necessary in order to identify these isolates.

After an initial digest of the representative isolates from Pro-Avi® with *Hae*III, isolates belonging to RAPD groups 19, 25, and 27 were identified as *L. salivarius* (Figure A-16). Isolates from RAPD groups 21, 22, 24, 26, 30, 31, and 32 were observed to share a banding pattern with the *L. gasseri* reference strain (Figures A-16 and A-17), so an *Mse*I digestion was necessary to identify the isolates as either *Lactobacillus gasseri* or *L. johnsonii*. Based on the results of this digestion, isolates belonging to RAPD groups 21, 22, 24, 26, 30, 31, and 32 were identified as *L. johnsonii* (ATCC 33200) (Figure A-14). Isolates from RAPD groups 20, 28, 29, and 33 showed banding patterns similar to those commonly observed for *Enterococcus* species, so the *Hae*III digestion was repeated for the representative isolates for these RAPD groups and reference strains of *E. faecalis* and *E. faecium*. Isolates belonging to RAPD groups 20, 28, 29, and 33 were subsequently identified as *E. faecalis* (Figure A-15). No identification was possible for the Pro-Avi®

isolates from RAPD groups 18, 23, and 34 since their banding patterns were not similar to any of the reference strains used (Figures A-15 and A-16). Further comparisons to additional reference strains would be necessary to identify these isolates.

Although the manufacturer lists *L. bifidus* as a component of both Pro-Avi® and Interbac®, no bacterial species was found that could reasonably account for this. The name *L. bifidus* itself is outdated. This taxonomy was had been used up to the mid 1950's but from that time up to the present there have been 24 similar species described and grouped into a new genera: *Bifidobacterium* (Sgorbati *et al.*, 1995). The organism originally identified as *L. bifidus* has since reclassified as *Bifidobacterium bifidum* (Kandler and Weiss, 1986). Based on this reclassification, it was assumed that the reference to *L. bifidus* in Pro-Avi® and Interbac® likely referred to some species of the genus *Bifidobacterium*. However, this was not the case, since no species from that genus was isolated from either product using the techniques employed in the present study.

If there is in fact bifidobacteria in the products, there are a number of factors which could be responsible for the failure to isolate it, among which media selection is the most likely. Aside from BHI, as used in the present study, alternative media used to culture bifidobacteria include TOS and MCA; however, a poor recovery rate for bifidobacteria was also attained on these media (Temmerman *et al.*, 2003). MRS medium supplemented with lysine has been used to successfully culture bifidobacteria (Yeung *et al.,* 2002). Another possibility is that processing before freeze-drying of the product may have damaged the cells, so they were not in culturable form in the product. Exposure to oxygen during processing or storage could also cause the death of any *Bifidobacterium* species, since the members of this genus are highly sensitive to oxygen and only survive under anaerobic conditions. There have also been past instances identified where organisms sold to probiotic manufacturers as *L. bifidus* were actually identified as various *Lactobacillus* species after analysis (Hamilton-Miller *et al.*, 1999).

Bacteria are no longer metabolically active once they have been freeze-dried, so there is no risk of damaging or killing the cells through exposure to oxygen at this point. However, once the anaerobic bacteria are exposed to moisture (during reconstitution for Interbac®, and moisture in the feed for Pro-Avi®) they are once again vulnerable, and could be killed by exposure to oxygen. This raises concerns regarding the practicality of including strict anaerobes in probiotic products, which according to manufacturer instructions, are to be exposed to both moisture and oxygen prior to being administered. For a probiotic bacterial strain to exert a beneficial effect in the GIT, it must thrive in two drastically different environments: in the product, which may be exposed to air or water, and in the anaerobic environment of the GIT. If the bacteria do not survive long enough to colonize the GIT, the lysis of these cells releases substrates that can be degraded and used by the bird, or by other microorganisms in the GIT (Mead, 1997). However, live probiotic bacteria are required for the bird to benefit (Nurmi and Rantala, 1973).

Despite the fact that it was not listed on the label of either Interbac® or Pro-Avi®, *L. johnsonii* was isolated from both products. Since *L. johnsonii* is one of the *L. acidophilus* group of bacteria (Yeung *et al.*, 2002), it is possible that it was included rather than *L. acidophilus*, which was listed by the manufacturer, but not recovered from either product. *L. johnsonii* has been isolated from the GIT of chickens in the past (Hammes and Vogel, 1995), so its inclusion is in keeping with the goal of providing bacterial species in probiotics that are components of the commensal microflora of a healthy adult chicken. However, *L. johnsonii* has also be isolated from the GIT of pigs and humans so the source of the organism used in these products would still be important, due to the fact that the efficacy of probiotic bacteria is greatly increased when the bacterial strains used are host-specific. *L. acidophilus* and *L. johnsonii* are so closely related that they cannot be differentiated based on the use of phenotypic methods alone. Since phenotypic methods are widely used in industry, this may explain the common misidentification of species in this taxonomic group as *L. acidophilus* (Yeung *et al.*, 2002). Another potential explanation for the lactobacilli being commonly misidentified as *L. acidophilus* may be that manufacturers intentionally chose to label closely related organisms as *L. acidophilus* for sales purposes since consumers are more likely to recognize this species name (Yeung *et al.*, 2002).

*L. salivarius* was also isolated from Pro-Avi®, despite the fact that it is not identified as a component by the manufacturer. However, the exclusion of this species from the product labeling poses no threat to bird health, since *L. salivarius* has been isolated from the commensal microflora in the GIT of chickens (Hammes and Vogel, 1995). This species is in fact a predominant species in the crop early in the life of the bird, before the commensal microflora is fully developed (Guan *et al.*, 2003). As with *L. johnsonii*, the issue of source would also be important with *L. salivarius*, since it has also been isolated form the GIT of other animals.

*E. faecalis* was isolated from both Pro-Avi® and Interbac®. This was the sole species listed by the manufacturer that was actually present and identified using current taxonomy in each product. *E. faecalis* has been found to be predominant in the GIT of young chicks, with decreasing numbers as the bird ages (Devrise and Pot, 1995). This makes it an ideal candidate for inclusion in products such as Pro-Avi® and Interbac®, which are designed to be administered shortly after hatching, and to encourage the initial development of a healthy GIT microflora.

Isolates from RAPD group 10 (Interbac®) could not be identified. After enzyme digestion, no banding patterns were observed for the isolate chosen as a representative of RAPD group 10. This occurred even though there was a product successfully amplified after ARDRA-PCR (data not shown). Future analysis may include repeating the ARDRA-PCR and enzyme digestion with a different isolate from RAPD group 10, or sequencing the 16S rDNA gene and comparing it to sequences for known type strain DNA.

Isolates for RAPD groups 18, 23, and 34 (Pro-Avi®) were also unidentifiable using the techniques employed in the present study. These three RAPD groups did share a common banding pattern after ARDRA analysis (Figure A-15), indicating that they are probably all of the same species. The banding pattern shared similarities with type strains of *Enterococcus* species, so it is theorized that these isolates belong to a different *Enterococcus* species than those tested in the present study, or perhaps to a *Pediococcus* species (G. Allison, personal communication). More comparisons to type strains are necessary.

All organisms isolated from both Pro-Avi® and Interbac® are facultative anaerobes (Hammes and Vogel, 1995; Devrise and Pot, 1995), making them ideal as probiotic bacteria since they are capable of surviving two environments which differ drastically: an aerobic environment during product processing and potentially during product storage, and the anaerobic environment found within the chicken GIT. It is crucial that probiotic bacterial strains be able to survive, grow, and reproduce in these two environments; maintaining the viability of cultures in sufficient numbers both during and after processing is a constant struggle for manufacturers (Mattila-Sandholm *et al*, 2002).

It is also important to note that though the species contained in both Pro-Avi® and Interbac® were identified in the present study, it cannot be conclusively stated which exact strains were included. This is of great importance since probiotics are known to be most effective when they are composed of species-specific strains. In most cases, commensal bacterial strains isolated from the GIT of chickens are more likely to be able to attach to the epithelial cells on the walls of the chicken GIT (Brooker and Fuller, 1975). This host specificity is especially evident in lactobacilli, which have been shown to be unable to adhere to the epithelial cells of animals from which they have not been isolated (Haddadin *et al*., 1996; Cox *et al*., 2001). Different strains of the same species do not necessarily possess the same properties. Thus it is important that bacteria be identified not only by species, but by strain as well in order to determine their effects *in vivo* (Hamilton-Miller *et al*., 1999). The variety of RAPD PCR patterns, indicating different strains, generated by isolates later shown to belong to only a few species, indicates that more than one strain of each species identified may be present in these probiotic products. Future experiments would be necessary in order to identify these specific strains and determine whether these same strains can be isolated from the GIT of broiler chickens after administration of the probiotic products.

## Conclusions and Recommendations for Future Analyses

Although the CFU/g of both Pro-Avi® and Interbac exceeded the minimum effective dose (Pascual *et al.*, 1999) and the manufacturer's claims, there were some qualitative inconsistencies. Both products lacked species listed on the label, while containing species not listed on the label, as well as species that were not identified in the present study. These findings warrant the use of other microbiological and molecular biology techniques to further evaluate these products before any further broiler trials. Further analysis would provide a more comprehensive picture of the bacterial populations in these probiotic products.

Before any further broiler trials are conducted, it is strongly recommended that the complete characterization of these products be completed; this should include the identification of species not identified in the present study, and the determination of the exact strain or strains of each species present. Once all isolates have been identified at the species and strain levels, then the available literature can be reviewed to assess what is known about the effects of these strains *in vivo* in the chicken GIT. Once the effects of the strains included in Interbac® and Pro-Avi® are better known, more appropriate animal trials can be designed to look specifically at those physiological characteristics most likely to be affected by the products. Trials to compare the GIT bacterial populations of both probiotic supplemented and unsupplemented broilers would also be valuable in assessing the ability of the bacteria in Pro-Avi® and Interbac® to persist in the GIT.

**TABLE A-1. Summary of original media and aeration conditions, target organisms, restreaking media and aeration conditions, and stock solution media used to isolate colonies from Pro-Avi® and Interbac®**

| Original Medium | Original Aeration | Target Organisms | Restreaking Medium | Restreaking Aeration | 50% Glycerol Stock |
|---|---|---|---|---|---|
| LBS Agar | anaerobic | *Lactobacillus* spp. | MRS Agar | anaerobic | MRS |
| MRS Agar | anaerobic | Other anaerobic lactic acid bacteria | MRS Agar | anaerobic | MRS |
| MRS Agar | aerobic | *Enterococcus* spp. | MRS Agar | aerobic | MRS |
| M17 Agar | aerobic | Other aerobic lactic acid bacteria (including enterococci) | MRS Agar | aerobic | MRS |
| BHI Agar | pre-reduced anaerobic | *Bifidobacterium* spp. and other strict anaerobes | BHI Agar | pre-reduced anaerobic | BHI |

**TABLE A-2. Reaction mixture components for Random Amplification of Polymorphic DNA (RAPD) PCR**

| Component | Volume (µl ) |
|---|---|
| DNA (1/20 or 1/50 dilution) | 1.0 |
| 10 mM dNTP[1] | 0.5 |
| 10X Tris-HCl buffer[1] (PCR reaction buffer) | 2.0 |
| 50mM MgCl2[1] | 1.0 |
| Taq polymerase[1] (5 units/ml) | 0.5 |
| OPA-02 primer [1] (5pmol/(µl) | 1.0 |
| sterile water | 14.0 |

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[1] Invitrogen Canada Inc., Burlington, ON, Canada, L7P 1A1

**TABLE A-3. Reaction mixture components for Amplified Ribosomal DNA Restriction Analysis (ARDRA) PCR**

| Component | Volume (µl ) |
|---|---|
| 1/20 diluted DNA | 1.0 |
| 10X buffer[1] | 5.0 |
| 50mM MgCl2[1] | 2.0 |
| 10 mM dNTP[1] | 1.0 |
| Lb16a primer[1] (25 pmol/ml) | 1.0 |
| 231B primer[1] (25 pmol/ml) | 1.0 |
| Taq polymerase[1] (5 units/ml) | 0.5 |
| sterile water | 38.5 |

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[1] Invitrogen Canada Inc., Burlington, ON, Canada, L7P 1A1

**TABLE A-4. Total numbers of lactic acid bacteria colony forming units per gram of Pro-Avi® and Interbac® on different media under different aeration conditions**

| Product | Anaerobic MRS (CFU/g) | Anaerobic LBS (CFU/g) | Anaerobic BHI (CFU/g) | Aerobic MRS (CFU/g) | Aerobic M17 (CFU/g) |
|---|---|---|---|---|---|
| Pro-Avi® | $3.40 \times 10^7$ | $6.00 \times 10^5$ | $5.90 \times 10^7$ | $3.70 \times 10^7$ | $3.60 \times 10^7$ |
| Interbac® | $2.74 \times 10^{11}$ | $5.10 \times 10^{10}$ | $1.54 \times 10^{11}$ | $1.85 \times 10^{11}$ | $1.65 \times 10^{11}$ |

**TABLE A-5. Theoretical and actual doses of Pro-Avi® and Interbac® administered to broiler chickens in the trials described in this thesis**

| Probiotic Treatment | Method of Administration | Actual Dose (CFU/ bird)[1] | Theoretical Dose (CFU/ bird)[1] |
|---|---|---|---|
| Interbac® | Drinking Water | $5.04 \times 10^3$ | $7.12 \times 10^6$ |
| Interbac® | Coarse Spray | $2.2 \times 10^5$ | $4.27 \times 10^7$ |
| Pro-Avi® | Feed Additive | $6.41 \times 10^7$ | $6.41 \times 10^7$ |
| Negative Control | Water and Spray | <1000 | <1000 |
| Negative Control | Feed | $1.05 \times 10^7$ | <1000 |

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[1] All doses calculated based on the number of CFU cultured on anaerobically incubated MRS agar plates.

**TABLE A-6. Identification codes and characteristics of colonies selected from Interbac® and Pro-Avi® for further study**

| Probiotic Product | Colony Type | Colonies Sampled | Medium | Incubation Conditions | Dilution | Colony Morphology |
|---|---|---|---|---|---|---|
| Interbac® | A | A-1, A-2, A-3 | MRS | anaerobic | $10^{-7}$ | large, smooth, opaque, center more opaque than edges |
| Interbac® | B | B-1 | MRS | anaerobic | $10^{-7}$ | large, opaque, fuzzy |
| Interbac® | C | C-1, C-2, C-3 | MRS | anaerobic | $10^{-7}$ | small, opaque, smooth, round |
| Interbac® | D | D-1, D-2, D-3 | MRS | anaerobic | $10^{-7}$ | medium, opaque, smooth, round |
| Interbac® | E | E-1, E-2, E-3 | MRS | anaerobic | $10^{-6}$ | very small, round, translucent |
| Interbac® | F | F-1, F-2, F-3 | LBS | anaerobic | $10^{-6}$ | medium, opaque, smooth, round |
| Interbac® | G | G-1, G-2, G-3 | LBS | anaerobic | $10^{-6}$ | large, very fuzzy, translucent, irregular edges |
| Interbac® | H | H-1, H-2, H-3 | LBS | anaerobic | $10^{-6}$ | very small, round, fuzzy, opaque |
| Interbac® | I | I-1, I-2, I-3 | LBS | anaerobic | $10^{-6}$ | very small, smooth, translucent, round |
| Interbac® | J | J-1, J-2 | LBS | anaerobic | $10^{-7}$ | small, irregular edges, opaque, fuzzy |
| Interbac® | V | V-1, V-2, V-3 | M17 | aerobic | $10^{-7}$ | medium, opaque center, irregular edges |
| Interbac® | W | W-1, W-2, W-3 | MRS | aerobic | $10^{-7}$ | medium, round, opaque, fuzzy surface |
| Interbac® | X | X-1, X-2, X-3 | MRS | aerobic | $10^{-7}$ | medium, transluscent, fuzzy surface |
| Interbac® | Y | Y-1, Y-2, Y-3 | MRS | aerobic | $10^{-7}$ | very small, opaque, smooth, round |
| Interbac® | B' | B'-1, B'-2,B'-3 | BHI | anaerobic | $10^{-8}$ | large, opaque center, fuzzy, translucent edges |
| Interbac® | C' | C'-1, C'-2, C'-3 | BHI | anaerobic | $10^{-5}$ | medium, opaque center, irregular edges, translucent edges |
| Interbac® | D' | D'-1, D'-2, D'-3 | BHI | anaerobic | $10^{-6}$ | very small, opaque, fuzzy |
| Pro-Avi® | K | K-1, K-2, K-3 | MRS | anaerobic | $10^{-3}$ | large, smooth white, round |
| Pro-Avi® | L | L-1, L-2, L-3 | MRS | anaerobic | $10^{-3}$ | small, smooth, white, round |
| Pro-Avi® | M | M-1, M-2, M-3 | MRS | anaerobic | $10^{-3}$ | small, fuzzy, white, more white in center than edges, irregular edges |
| Pro-Avi® | N | N-1, N-2, N-3 | LBS | anaerobic | $10^{-2}$ | large, white, smooth, round |
| Pro-Avi® | O | O-1, O-2 | LBS | anaerobic | $10^{-2}$ | very small, white, smooth, round |
| Pro-Avi® | P | P-1, P-2, P-3 | LBS | anaerobic | $10^{-2}$ | small, fuzzy, white in center, irregular edges |
| Pro-Avi® | R | R-1, R-2, R-3, R-4 | BHI | anaerobic | $10^{-5}$ | very small, smooth, round, white |
| Pro-Avi® | S | S-1, S-2, S-3 | MRS | aerobic | $10^{-3}$ | medium, smooth, round, white |
| Pro-Avi® | T | T-1, T-2, T-3 | MRS | aerobic | $10^{-3}$ | large, smooth, round, white |
| Pro-Avi® | U | U-1, U-2, U-3 | MRS | aerobic | $10^{-3}$ | medium, opaque, fuzzy edges |
| Pro-Avi® | Z | Z-1, Z-2, Z-3 | M17 | aerobic | $10^{-4}$ | medium, smooth, round, white |
| Pro-Avi® | A' | A'-1, A'-2, A'-3 | M17 | aerobic | $10^{-3}$ | medium, smooth, round, opaque |

**TABLE A-7. Representative isolates, isolates included in RAPD groups, and species identifications based on ARDRA-PCR for Pro-Avi® and Interbac®**

| Product | Group | Representative Colony | Colonies Included | Species Identification Based on ARDRA |
|---|---|---|---|---|
| Interbac® | 1 | A-3 | A-1, A-2, A-3 | *L. johnsonii* (ATCC 33200) |
| Interbac® | 2 | C-2 | B-1, C-1, C-2, C-3, F-1, F-3, G-1, H-1, H-3 | *L. johnsonii* (ATCC 33200) |
| Interbac® | 3 | H-2 | E-1, E-3, H-2, I-2, I-3, J-1, J-2 | *L. johnsonii* (ATCC 33200) |
| Interbac® | 4 | I-1 | G-2, G-3, E-2, I-1, F-2 | *L. johnsonii* (ATCC 33200) |
| Interbac® | 5 | D-2A | D-1, D-2A[1], D-2B[1] | *L. johnsonii* (ATCC 33200) |
| Interbac® | 6 | D-3 | D-3 | *L. johnsonii* (ATCC 33200) |
| Interbac® | 7 | V-1 | V-1, V-2, V-3, W-1 | *L. johnsonii* (ATCC 33200) |
| Interbac® | 8 | W-2 | W-2 | *L. johnsonii* (ATCC 33200) |
| Interbac® | 9 | W-3 | W-3 | *L. johnsonii* (ATCC 33200) |
| Interbac® | 10 | X-1 | X-1, X-2 | unidentified |
| Interbac® | 11 | X-3 | X-3 | *L. johnsonii* (ATCC 33200) |
| Interbac® | 12 | Y-1 | Y-1 | *L. johnsonii* (ATCC 33200) |
| Interbac® | 13 | Y-2 | Y-2 | *L. johnsonii* (ATCC 33200) |
| Interbac® | 14 | Y-3 | Y-3 | *L. johnsonii* (ATCC 33200) |
| Interbac® | 15 | B'-2 | B'-1, B'-2, B'-3, D'-1, D'-2, D'-3 | *L. johnsonii* (ATCC 33200) |
| Interbac® | 16 | C'-1 | C'-1 | *E. faecalis* (ATCC 19433) |
| Interbac® | 17 | C'-2 | C'-2, C'-3 | *E. faecalis* (ATCC 19433) |
| Pro-Avi® | 18 | K-2 | K-1, K-2, K-3 | unidentified |
| Pro-Avi® | 19 | L-1 | L-1 | *L. salivarius* (ATCC 11741) |
| Pro-Avi® | 20 | L-3 | L-2, L-3 | *E. faecalis* (ATCC 19433) |
| Pro-Avi® | 21 | M-2 | M-2 | *L. johnsonii* (ATCC 33200) |
| Pro-Avi® | 22 | M-3 | M-3 | *L. johnsonii* (ATCC 33200) |
| Pro-Avi® | 23 | N-2 | N-1, N-2, N-3, O-1, O-2 | unidentified |
| Pro-Avi® | 24 | P-3 | P-1, P-2, P-3 | *L. johnsonii* (ATCC 33200) |
| Pro-Avi® | 25 | R-2 | R-1, R-2, R-3 | *L. salivarius* (ATCC 11741) |
| Pro-Avi® | 26 | M-1 | M-1 | *L. johnsonii* (ATCC 33200) |
| Pro-Avi® | 27 | R-4 | R-4 | *L. salivarius* (ATCC 11741) |
| Pro-Avi® | 28 | S-3 | S-1, S-2, S-3, Z-1, Z-2 | *E. faecalis* (ATCC 19433) |
| Pro-Avi® | 29 | T-2 | T-1, T-2, T-3, U-1, A'-1, A'-2 | *E. faecalis* (ATCC 19433) |
| Pro-Avi® | 30 | U-2 | U-2 | *L. johnsonii* (ATCC 33200) |
| Pro-Avi® | 31 | U-3A | U-3A[1] | *L. johnsonii* (ATCC 33200) |
| Pro-Avi® | 32 | U-3B | U-3B[1] | *L. johnsonii* (ATCC 33200) |
| Pro-Avi® | 33 | Z-3 | Z-3 | *E. faecalis* (ATCC 19433) |
| Pro-Avi® | 34 | A'-3 | A'-3 | unidentified |

[1] D-2A and D-2B are two different colony types which were observed when colony D-2 was originally streaked, providing an impure culture. Likewise, U-3A and U-3B were isolated from an impure culture after the original streaking of colony U-3.

**FIGURE A-1. Summary of the procedure used in the microbiological analysis of Pro-Avi® and Interbac®**

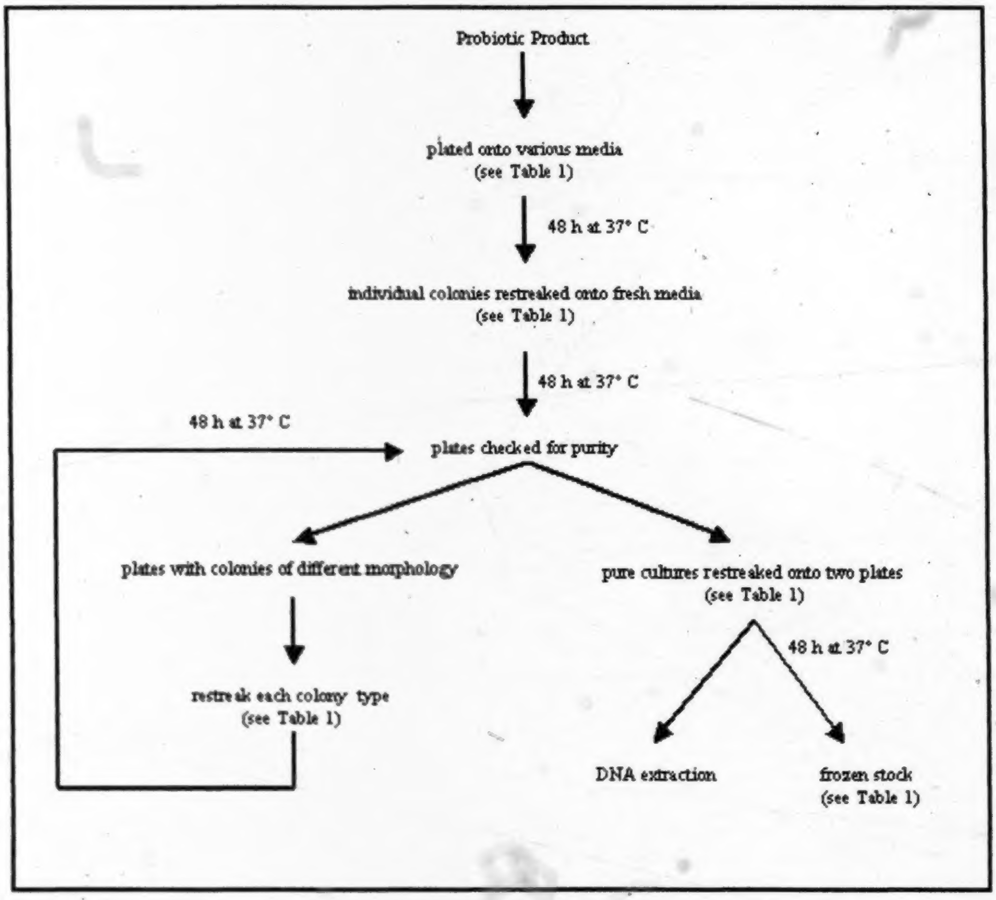

Probiotic Product
plated onto various media
(see Table 1)
48 h at 37° C
individual colonies restreaked onto fresh media
(see Table 1)
48 h at 37° C
48 h at 37° C
plates checked for purity
plates with colonies of different morphology
pure cultures restreaked onto two plates
(see Table 1)
48 h at 37° C
restreak each colony type
(see Table 1)
DNA extraction
frozen stock
(see Table 1)

**FIGURE A-2. The time and temperature profile used for RAPD PCR**

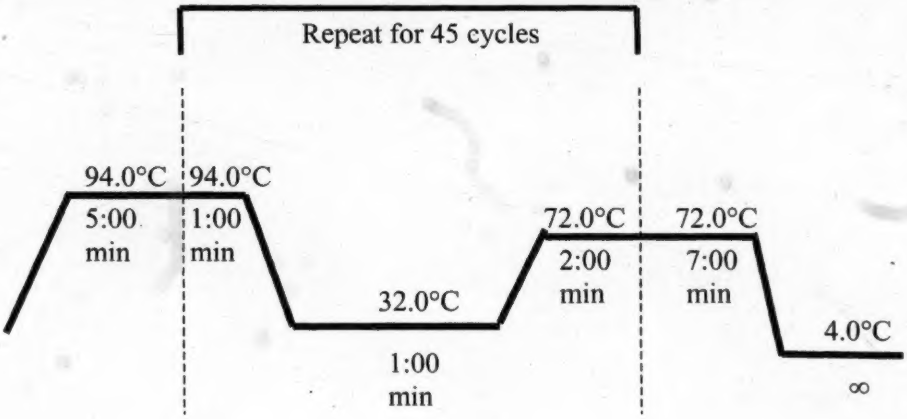

Repeat for 45 cycles
94.0°C
5:00
min
94.0°C
1:00
min
32.0°C
1:00
min
72.0°C
2:00
min
72.0°C
7:00
min
4.0°C
∞

FIGURE A-3. The time and temperature profile used for ARDRA-PCR

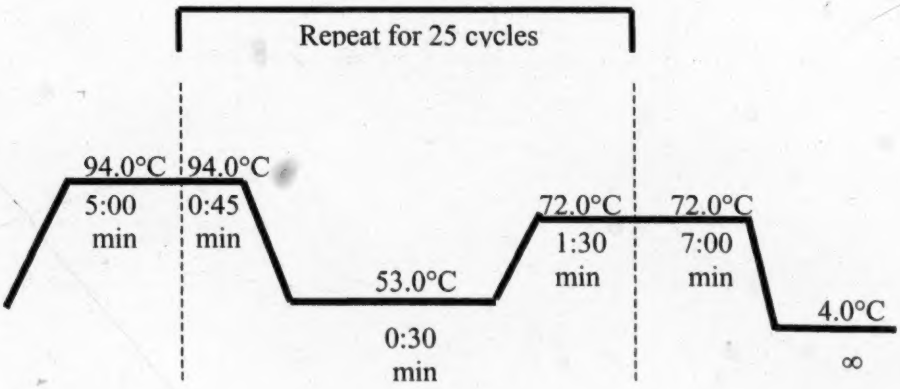

Repeat for 25 cycles
94.0°C
5:00
min
94.0°C
0:45
min
53.0°C
0:30
min
72.0°C
1:30
min
72.0°C
7:00
min
4.0°C
∞

**FIGURE A-4. RAPD PCR results for isolates from Interbac®.** RAPD groups as well as the representative isolates are indicated above each lane. The lane containing the 1 kb ladder is indicated by "ldr." The sizes of the DNA fragments in the ladder are indicated along the side of the gel. Four distinctive banding patterns, indicating four RAPD groups, can be seen.

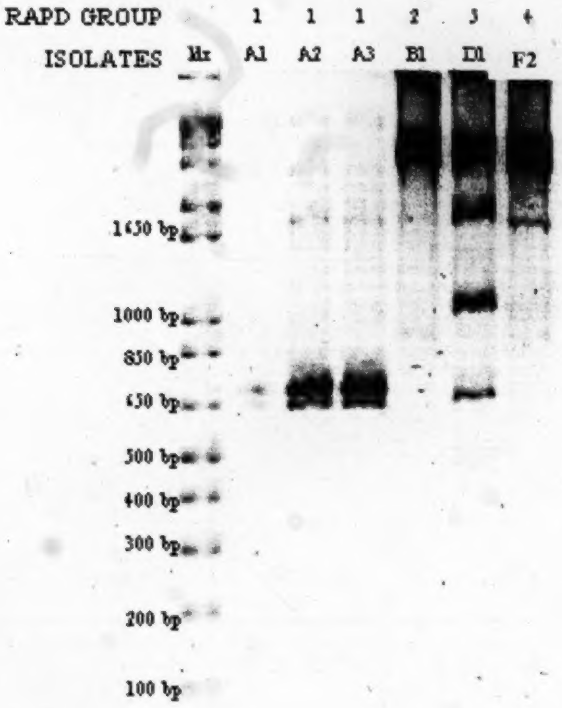

RAPD GROUP
1
1
1
2
3
4
ISOLATES
Mr
A1
A2
A3
B1
D1
F2
1650 bp
1000 bp
850 bp
650 bp
500 bp
400 bp
300 bp
200 bp
100 bp

**FIGURE A-5. RAPD PCR results for isolates from Interbac®.** DNA dilutions were adjusted prior to PCR. RAPD groups as well as the representative isolates are indicated above each lane. The lane containing the 1 kb ladder is indicated by "ldr." The sizes of the DNA fragments in the ladder are indicated along the side of the gel. Two distinctive banding patterns, indicating two RAPD groups, can be seen.

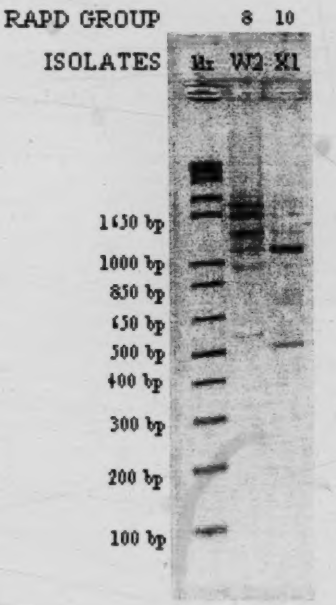

RAPD GROUP
8
10
ISOLATES
ldr
W2
X1
1650 bp
1000 bp
850 bp
650 bp
500 bp
400 bp
300 bp
200 bp
100 bp

**FIGURE A-6. RAPD PCR results for isolates from Interbac®.** RAPD groups as well as the representative isolates are indicated above each lane. The lane containing the negative control is indicated by "ctl", while the lane containing the 1 kb ladder is indicated by "ldr." The sizes of the DNA fragments in the ladder are indicated along the side of the gel. D-2A and D-2B are different colony types that resulted when colony D-2 was originally streaked, resulting in an impure culture with two distinctive colony types. Three distinctive banding patterns, indicating three RAPD groups, can be seen.

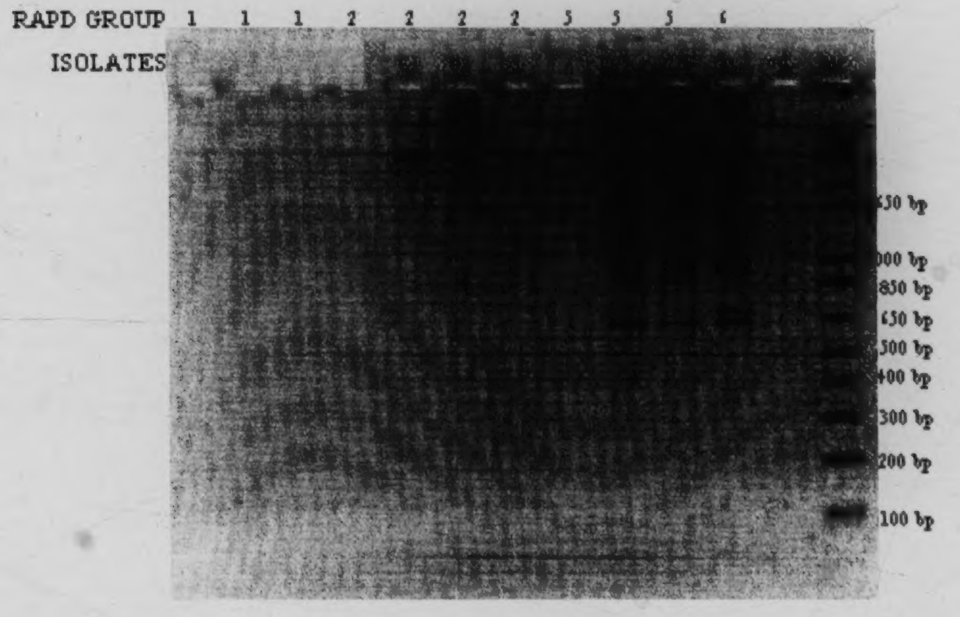

RAPD GROUP 1 1 1 2 2 2 2 3 3 3 6
ISOLATES
650 bp
000 bp
850 bp
650 bp
500 bp
400 bp
300 bp
200 bp
100 bp

**FIGURE A-7. RAPD PCR results for isolates from Interbac®.** RAPD groups as well as the representative isolates are indicated above each lane. The lane containing the negative control is indicated by "ctl", while the lane containing the 1 kb ladder is indicated by "ldr." The sizes of the DNA fragments in the ladder are indicated along the side of the gel. In this gel the presence of a contaminant can be seen in the lane containing the negative control. However, the bands seen in the negative control did not interfere with the ability to group the isolates into RAPD groups, so these bands were simply ignored. This was the only gel in which any contamination was seen in the lane containing the negative control. Three distinctive banding patterns, indicating three RAPD groups, can be seen.

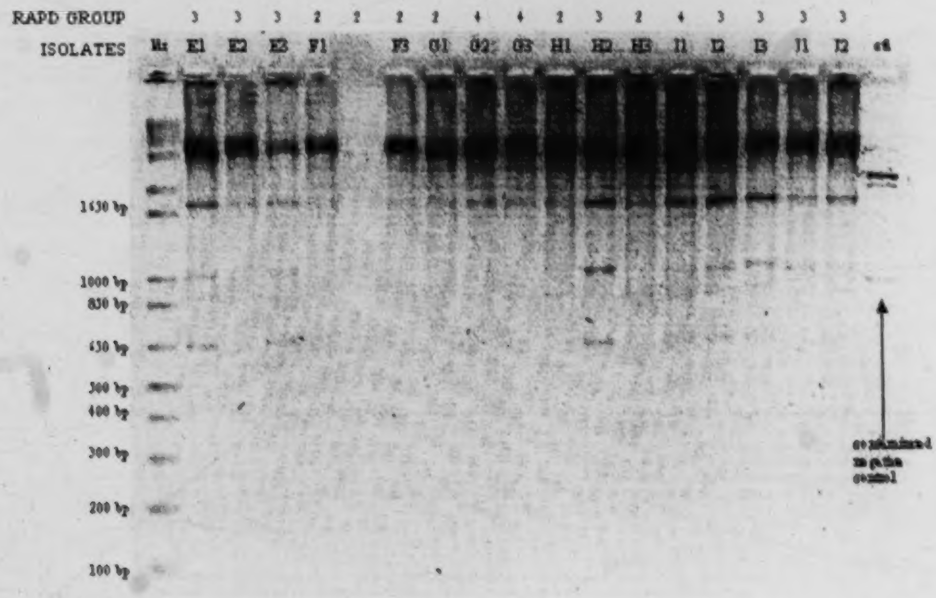

RAPD GROUP
3 3 3 2 2 2 2 4 4 2 3 2 4 3 3 3 3
ISOLATES
ldr E1 E2 E3 F1 F3 G1 G2 G3 H1 H2 H3 I1 I2 I3 J1 J2 ctl
1650 bp
1000 bp
850 bp
650 bp
500 bp
400 bp
300 bp
200 bp
100 bp
contaminated negative control

**FIGURE A-8. RAPD PCR results for isolates from Interbac®.** RAPD groups as well as the representative isolates are indicated above each lane. The lane containing the 1 kb ladder is indicated by "ldr." The sizes of the DNA fragments in the ladder are indicated along the side of the gel. Ten distinctive banding patterns, indicating ten RAPD groups, can be seen.

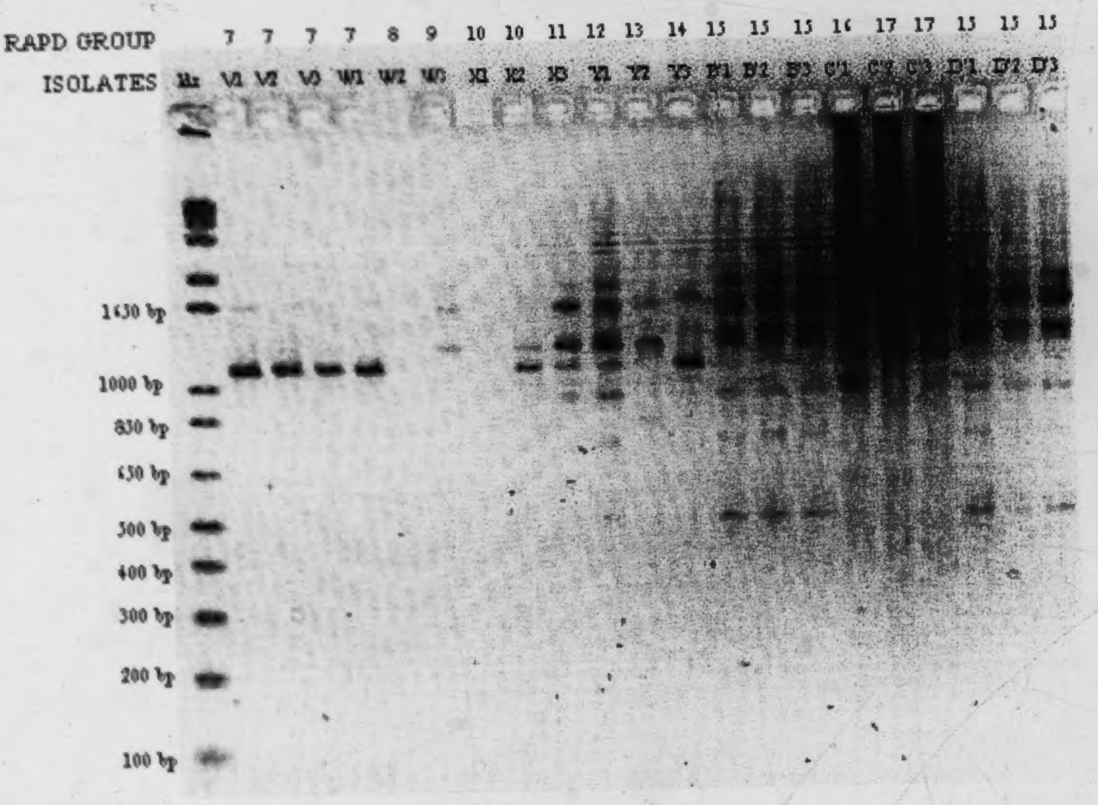

RAPD GROUP
7 7 7 7 8 9 10 10 11 12 13 14 15 15 15 16 17 17 15 15 15
ISOLATES
ldr V1 V2 V3 W1 W2 W3 X1 X2 X3 Y1 Y2 Y3 B1 B2 B3 C1 C2 C3 D1 D2 D3
1650 bp
1000 bp
850 bp
650 bp
500 bp
400 bp
300 bp
200 bp
100 bp

**FIGURE A-9. RAPD PCR results for isolates from Pro-Avi®.** RAPD groups as well as the representative isolates are indicated above each lane. The lane containing the 1 kb ladder is indicated by "ldr." The sizes of the DNA fragments in the ladder are indicated along the side of the gel. Ten distinctive banding patterns, indicating ten RAPD groups, can be seen.

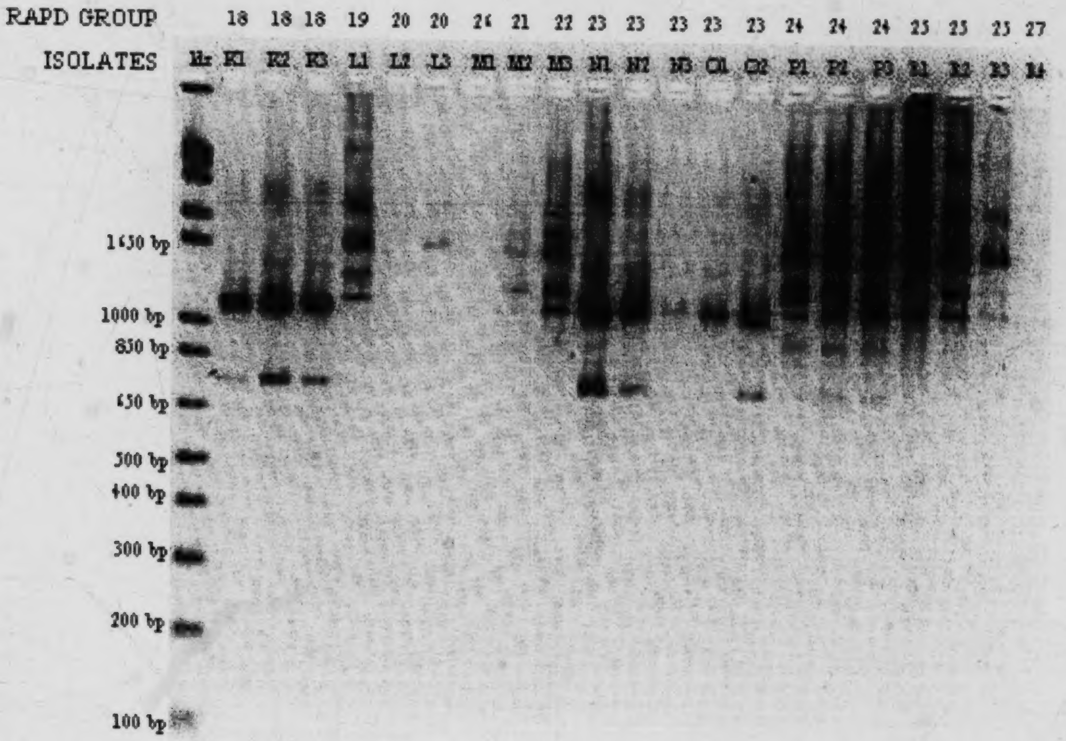

RAPD GROUP
18 18 18 19 20 20 21 21 22 23 23 23 23 23 24 24 24 25 25 25 27
ISOLATES
Ldr K1 K2 K3 L1 L2 L3 M1 M2 M3 N1 N2 N3 O1 O2 P1 P2 P3 R1 R2 R3 R4
1650 bp
1000 bp
850 bp
650 bp
500 bp
400 bp
300 bp
200 bp
100 bp

**FIGURE A-10. RAPD PCR results for isolates from Pro-Avi®.** RAPD groups as well as the representative isolates are indicated above each lane. The lane containing the 1 kb ladder is indicated by "ldr." The sizes of the DNA fragments in the ladder are indicated along the side of the gel. Seven distinctive banding patterns, indicating seven RAPD groups, can be seen.

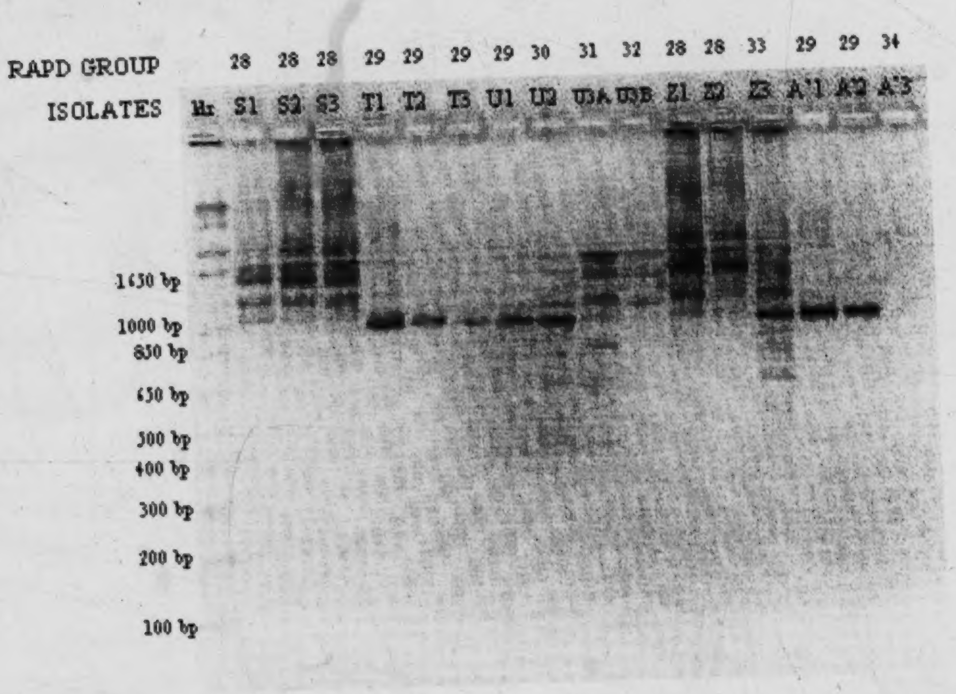

RAPD GROUP
28 28 28 29 29 29 29 30 31 32 28 28 33 29 29 34
ISOLATES
ldr S1 S2 S3 T1 T2 T3 U1 U2 U3A U3B Z1 Z2 Z3 A'1 A'2 A'3
1650 bp
1000 bp
850 bp
650 bp
500 bp
400 bp
300 bp
200 bp
100 bp

**FIGURE A-11. ARDRA PCR results for a *Hae*III digest of representative isolates from Interbac®.** RAPD group numbers and reference strain abbreviations are indicated above each lane. The identities of each reference strain are listed in the legend to the right of the gel. The lane containing the 1 kb ladder is indicated by "ldr." The sizes of the DNA fragments in the ladder are indicated along the side of the gel. All RAPD groups tested were either *L. johnsonii* or *L. gasseri*. An enzyme digestion with *Mse*I was needed to differentiate between the two species.

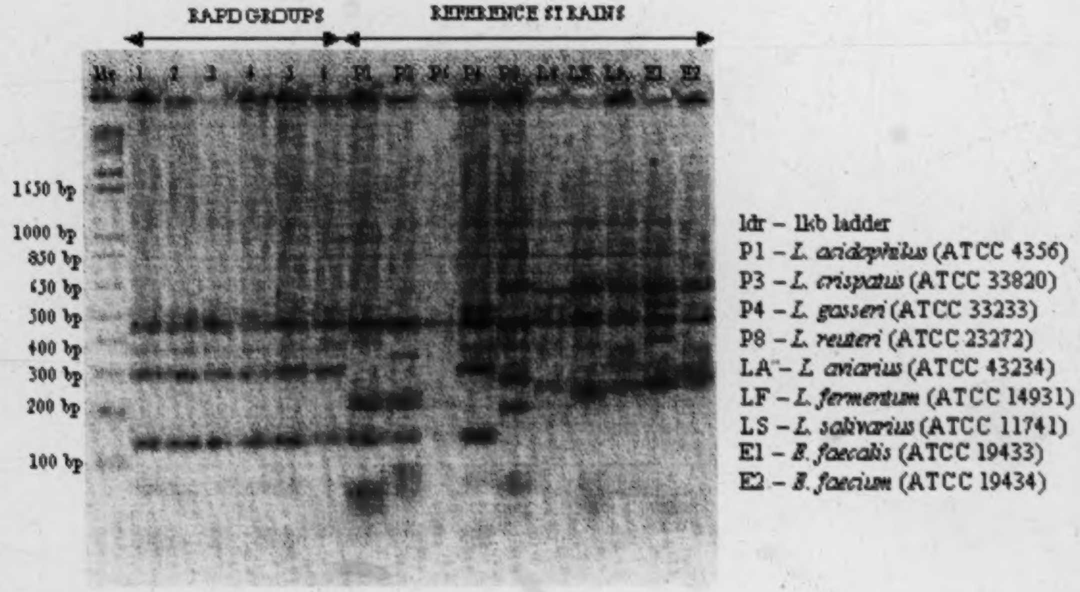
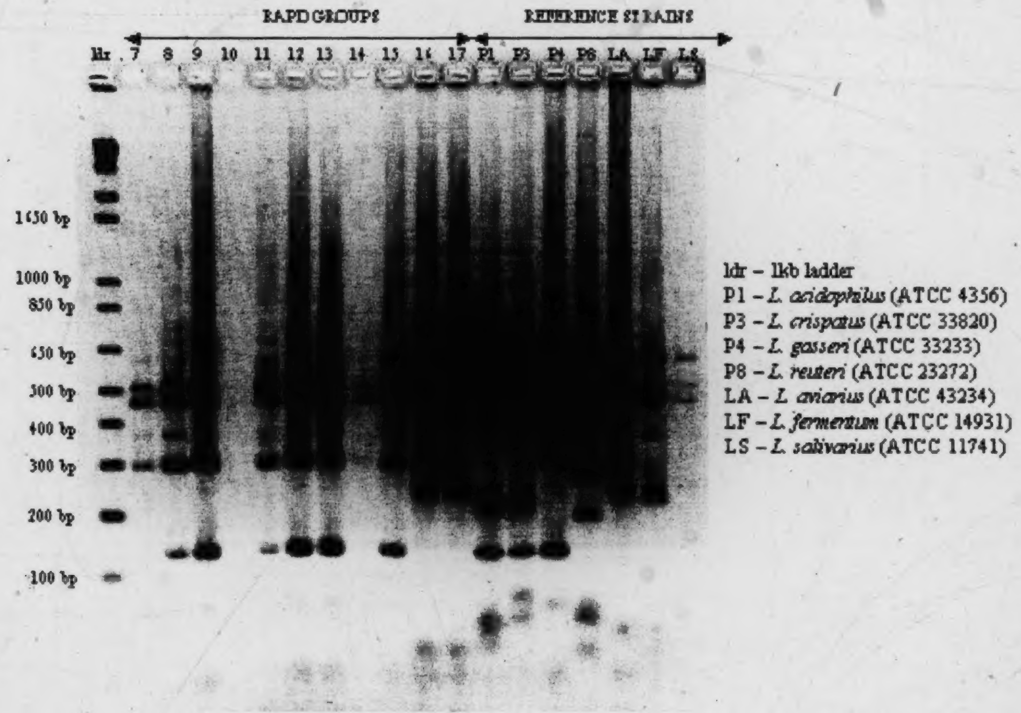

RAPD GROUPS
REFERENCE STRAINS
ldr
1
2
3
4
5
6
P1
E1
E2
1650 bp
1000 bp
850 bp
650 bp
500 bp
400 bp
300 bp
200 bp
100 bp
ldr – 1kb ladder
P1 – L. acidophilus (ATCC 4356)
P3 – L. crispatus (ATCC 33820)
P4 – L. gasseri (ATCC 33233)
P8 – L. reuteri (ATCC 23272)
LA – L. aviarius (ATCC 43234)
LF – L. fermentum (ATCC 14931)
LS – L. salivarius (ATCC 11741)
E1 – E. faecalis (ATCC 19433)
E2 – E. faecium (ATCC 19434)

**FIGURE A-12. ARDRA PCR results for a *Hae*III digest of representative isolates from Interbac®.** RAPD group numbers and reference strain abbreviations are indicated above each lane. The identities of each reference strain are listed in the legend to the right of the gel. The lane containing the 1 kb ladder is indicated by "ldr." The sizes of the DNA fragments in the ladder are indicated along the side of the gel. RAPD groups 7, 8, 9, 11, 12, 13, 14, and 15 were shown to be either *L. johnsonii* or *L. gasseri*. An enzyme digestion with *Mse*I was needed to differentiate between the two species. RAPD groups 10, 16, and 17 were not identified based on this gel.


RAPD GROUPS
REFERENCE STRAINS
ldr
7
8
9
10
11
12
13
14
15
16
17
P1
P3
P4
P8
LA
LF
LS
1650 bp
1000 bp
850 bp
650 bp
500 bp
400 bp
300 bp
200 bp
100 bp

ldr – 1kb ladder
P1 – *L. acidophilus* (ATCC 4356)
P3 – *L. crispatus* (ATCC 33820)
P4 – *L. gasseri* (ATCC 33233)
P8 – *L. reuteri* (ATCC 23272)
LA – *L. aviarius* (ATCC 43234)
LF – *L. fermentum* (ATCC 14931)
LS – *L. salivarius* (ATCC 11741)

**FIGURE A-13. ARDRA PCR results for a *Mse*I digest of representative isolates from Interbac®.** RAPD group numbers and reference strain abbreviations are indicated above each lane. The identities of each reference strain are listed in the legend to the right of the gel. The lane containing the 1 kb ladder is indicated by "ldr." The sizes of the DNA fragments in the ladder are indicated along the side of the gel. All RAPD groups tested were shown to be *L. johnsonii*.

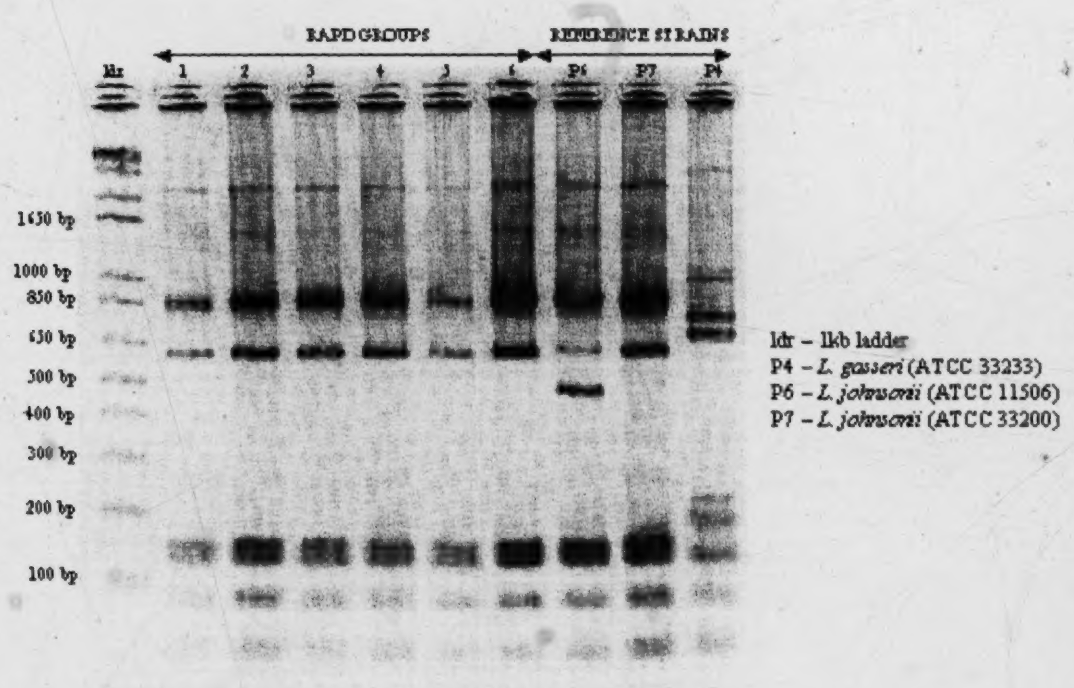
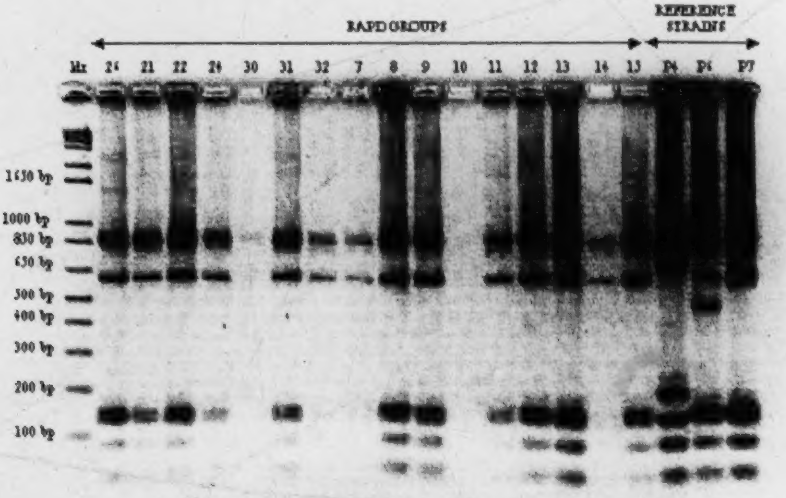

RAPD GROUPS
REFERENCE STRAINS
ldr
1
2
3
4
5
6
P6
P7
P4
1650 bp
1000 bp
850 bp
650 bp
500 bp
400 bp
300 bp
200 bp
100 bp
ldr – 1kb ladder
P4 – *L. gasseri* (ATCC 33233)
P6 – *L. johnsonii* (ATCC 11506)
P7 – *L. johnsonii* (ATCC 33200)

**FIGURE A-14. ARDRA PCR results for a *Mse*I digest of representative isolates from Interbac® and Pro-Avi®.** RAPD group numbers and reference strain abbreviations are indicated above each lane. The identities of each reference strain are listed in the legend to the right of the gel. The lane containing the 1 kb ladder is indicated by "ldr." The sizes of the DNA fragments in the ladder are indicated along the side of the gel. All RAPD groups tested, with the exception of RAPD group 10, were shown to be *L. johnsonii*. RAPD group 10 was not identified in this gel.


RAPD GROUPS
REFERENCE STRAINS
ldr
20
21
22
24
30
31
32
7
8
9
10
11
12
13
14
15
P4
P6
P7
1650 bp
1000 bp
850 bp
650 bp
500 bp
400 bp
300 bp
200 bp
100 bp

ldr – 1kb ladder
P4 – *L. gasseri* (ATCC 33233)
P6 – *L. johnsonii* (ATCC 11506)
P7 – *L. johnsonii* (ATCC 33200)

**FIGURE A-15. ARDRA PCR results for a *Hae*III digest of representative isolates from Interbac® and Pro-Avi®.** RAPD group numbers and reference strain abbreviations are indicated above each lane. The identities of each reference strain are listed in the legend to the right of the gel. The lane containing the 1 kb ladder is indicated by "ldr." The sizes of the DNA fragments in the ladder are indicated along the side of the gel. RAPD groups 16, 17, 20, 29, and 33 were shown to be *E. faecalis*. RAPD groups 10, 18, 23, and 34 were not identified in this gel.

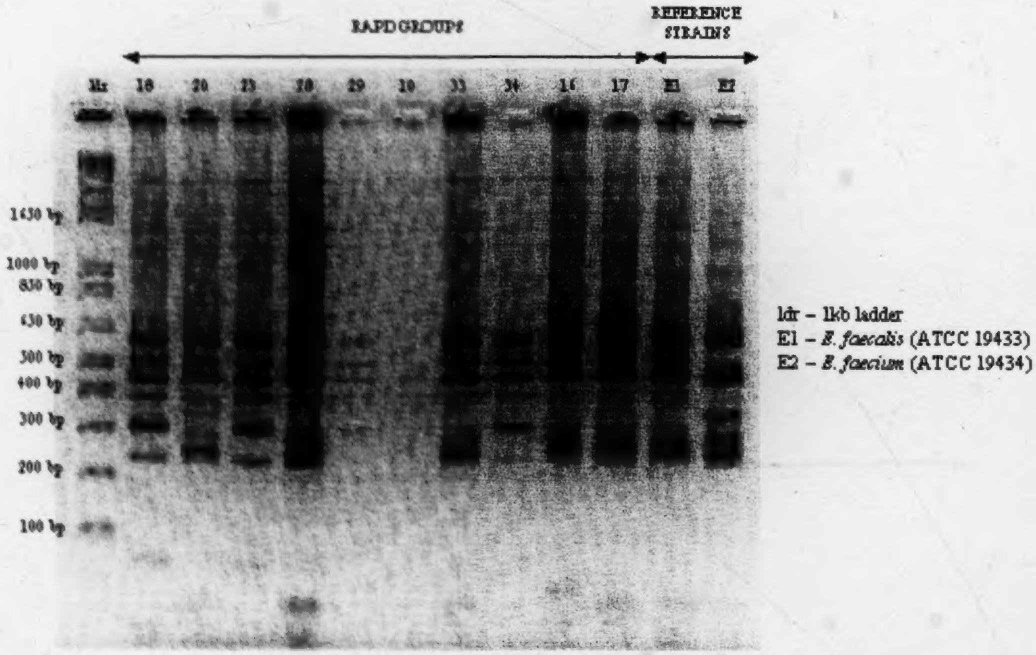

RAPD GROUPS
REFERENCE STRAINS
ldr
18
20
23
10
29
10
33
34
16
17
E1
E2
1650 bp
1000 bp
850 bp
650 bp
500 bp
400 bp
300 bp
200 bp
100 bp
ldr – 1kb ladder
E1 – E. faecalis (ATCC 19433)
E2 – E. faecium (ATCC 19434)

**FIGURE A-16. ARDRA PCR results for a *Hae*III digest of representative isolates from Pro-Avi®.** RAPD group numbers and reference strain abbreviations are indicated above each lane. The identities of each reference strain are listed in the legend to the right of the gel. The lane containing the 1 kb ladder is indicated by "ldr." The sizes of the DNA fragments in the ladder are indicated along the side of the gel. No banding pattern was observed for *L. salivarius* in this particular gel, so the *L. salivarius* banding pattern and corresponding ladder from another gel are shown at the right. RAPD groups 19, 25, and 27 were identified as *L. salivarius*. RAPD groups 21, 22, 24, and 26 were shown to be either *L. johnsonii* or *L. gasseri.* An enzyme digestion with *Mse*I was needed to differentiate between the two species. RAPD groups 18, 20, and 23 were not identified in this gel.

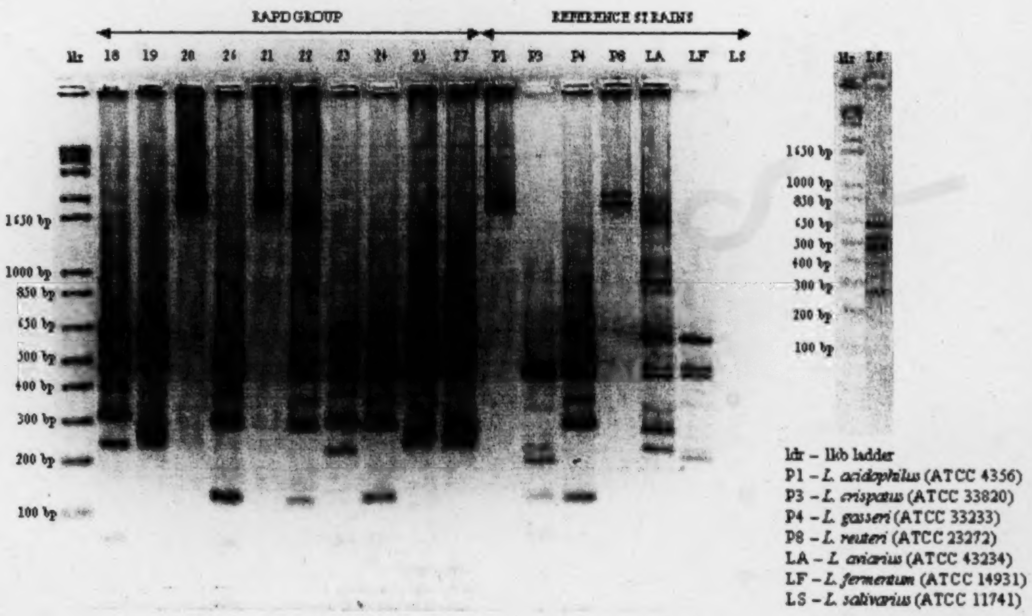
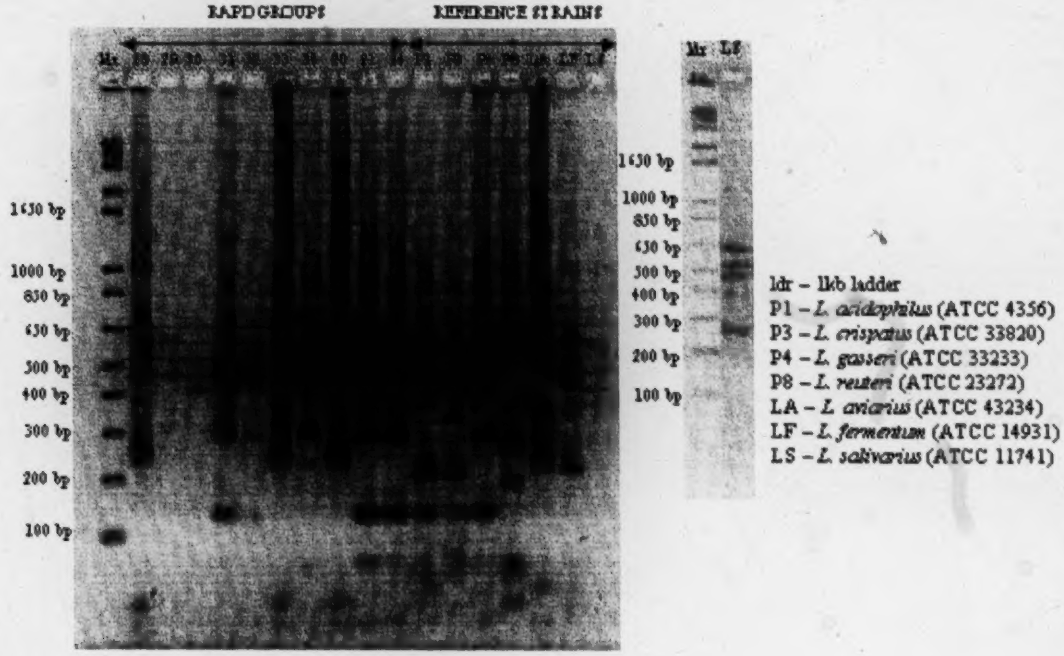

RAPD GROUP
REFERENCE STRAINS
ldr
18
19
20
26
21
22
23
24
25
27
P1
P3
P4
P8
LA
LF
LS
1650 bp
1000 bp
850 bp
650 bp
500 bp
400 bp
300 bp
200 bp
100 bp
ldr
LS
1650 bp
1000 bp
850 bp
650 bp
500 bp
400 bp
300 bp
200 bp
100 bp
ldr – 1kb ladder
P1 – L. acidophilus (ATCC 4356)
P3 – L. crispatus (ATCC 33820)
P4 – L. gasseri (ATCC 33233)
P8 – L. reuteri (ATCC 23272)
LA – L. aviarius (ATCC 43234)
LF – L. fermentum (ATCC 14931)
LS – L. salivarius (ATCC 11741)

**FIGURE A-17. ARDRA PCR results for a *Hae*III digest of representative isolates from Pro-Avi®.** RAPD group numbers and reference strain abbreviations are indicated above each lane. The identities of each reference strain are listed in the legend to the right of the gel. The lane containing the 1 kb ladder is indicated by "ldr." The sizes of the DNA fragments in the ladder are indicated along the side of the gel. No banding pattern was observed for *L. salivarius* in this particular gel, so the *L. salivarius* banding pattern and corresponding ladder from another gel are shown at the right. RAPD groups 21, 24, 30, 31, and 32 were shown to be either *L. johnsonii* or *L. gasseri.* An enzyme digestion with *Mse*I was needed to differentiate between the two species. RAPD groups 20, 28, 29, 33, and 34 were not identified in this gel.


RAPD GROUPS
REFERENCE STRAINS
1650 bp
1000 bp
850 bp
650 bp
500 bp
400 bp
300 bp
200 bp
100 bp
ldr
LS
1650 bp
1000 bp
850 bp
650 bp
500 bp
400 bp
300 bp
200 bp
100 bp
ldr – 1kb ladder
P1 – L. acidophilus (ATCC 4356)
P3 – L. crispatus (ATCC 33820)
P4 – L. gasseri (ATCC 33233)
P8 – L. reuteri (ATCC 23272)
LA – L. aviarius (ATCC 43234)
LF – L. fermentum (ATCC 14931)
LS – L. salivarius (ATCC 11741)

# REFERENCES

Allison, G., December 2003. personal communication. 2-50 Agriculture-Forestry Centre, Department of Agriculture, Food, and Nutritional Science, University of Alberta, Edmonton, AB, Canada, T6G 2P5.

Blankenship, L. C., J. S. Bailey, N. A. Cox, N. J. Stern, R. Brewer, and O. Williams, 1993. Two-step mucosal competitive exclusion flora treatment to diminish Salmonellae in commercial broiler chickens. Poult. Sci. 72:1667-1672.

Brooker, B. E. and R. Fuller, 1975. Adhesion of lactobacilli to the chicken crop epithelium. J. Ultrastructure Res. 52:21-31.

Canadian Animal Health Institute, 2003. Compendium of Veterinary Products, 8th Edition. North American Compendiums Ltd., Hensall, ON, Canada.

Cox, N. A., J. S. Bailey, and N. J. Stern, 2001. Effectiveness of an undefined mucosal competitive exclusion treatment to control *Salmonella* in turkeys during brooding. J. Appl. Poult. Res. 10:319-322.

Devriese, L. A., and B. Pot, 1995. The genus *Enterococcus*. Pages 327-367 in The Genera of Lactic Acid Bacteria. B. J. B. Wood and W. H. Holzapfel (ed.). Blackie Academic and Professional Press, London, UK.

Guan, L., December 2003. personal communication. 2-50 Agriculture-Forestry Centre, Department of Agriculture, Food, and Nutritional Science, University of Alberta, Edmonton, AB, Canada, T6G 2P5.

Guan, L., K. E. Hagen, G. W. Tannock, D. R. Korver, G. M. Fasenko, and G. E. Allison, 2003. Detection and identification of *Lactobacillus* species in crops of broilers of different ages by using PCR-denaturing gradient gel electrophoresis and amplified ribosomal DNA restriction analysis. Appl. Environ. Microbiol. 69:6750-6757.

Haddadin, M. S. Y., S. M. Abdulrahim, E. A. R. Hashlamoun, and R. K. Robinson, 1996. The effect of *Lactobacillus acidophilus* on the production and chemical composition of hen's eggs. Poult. Sci. 75:491-494.

Hamilton-Miller, J. M. T., S. Shah, and J. T. Winkler, 1999. Public health issues arising from microbiological and labeling quality of foods and supplements containing probiotic microorganisms. Public Health Nutr. 2:223-229.

Hammes, W. P., and R. F. Vogel, 1995. The genus *Lactobacillus*. Pages 19-54 in The Genera of Lactic Acid Bacteria. B. J. B. Wood and W. H. Holzapfel (ed.). Blackie Academic and Professional Press, London, UK.

Jin, L. Z., Y. W. Ho, N. Abdullah, and S. Jalaludin, 1998. Growth performance, intestinal microbial populations, and serum cholesterol of broilers fed diets containing *Lactobacillus* cultures. Poult. Sci. 77:1259-1265.

Jones, F.T., and K. E. Richardson, 2004. *Salmonella* in commercially manufactured feeds. Poult. Sci. 83:384-391.

Kandler, O., and N. Weiss, 1986. Regular nonsporing gram-positive rods. Pages 1208-1260 in Bergeys Manual of Systematic Bacteriology Vol. 2. P. Sneath, N. Mair, M. Sharpe, and J. Holt (ed). Williams and Wilkins, Baltimore, MD.

Madigan, M. T., J. M. Martinko, and J. Parker, 2003. Methods in microbial ecology. p. 614-631 *in:* Brock biology of microorganisms. 10th ed. Pearson Education Inc., Upper Saddle River, NJ.

Mattila-Sandholm, T., P. Myllärinen, R. Critteden, G. Mogensen, R. Fondén, and M. Saarela, 2002. Technological challenges for future probiotic foods. Int. Dairy J. 12:173-182.

Mead, G. C., 1997. Bacteria in the gastrointestinal tract of birds. Pages 216-240 in Gastroinstinal Microbiology Vol. II. R. Mackie, B. White, and R. Issacson (ed). Chapman and Hall, New York, NY.

Nurmi, E., and M. Rantala, 1973. New aspects in *Salmonella* infections in broiler production. Nature. 241:210-211.

Pascual, M., M. Hugas, J. I. Badiola, J. M. Monfort, and M. Garriga, 1999. *Lactobacillus salivarius* CTC2197 prevents *Salmonella enteritidis* colonization in chickens. Appl. Environ. Microbiol. 65:4981-4986.

Roy, D., P. Ward, D. Vincent, and F. Mondou, 2000. Molecular identification of potentially probiotic lactobacilli. Current Microbiol. 40:40-46.

Sambrook, J. and D. W. Russell, 2001a. Gel electrophoresis of DNA and pulsed-field agarose gel electrophoresis. Pages 5.1-5.90 in Molecular Cloning: A Laboratory Manual. 3rd Edition, Vol. I. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sambrook, J. and D. W. Russell, 2001b. Appendix 1: Preparation of reagents and buffers used in molecular cloning. Pages A1.2-A1.30 in Molecular Cloning: A Laboratory Manual. 3rd Edition, Vol. III. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sgorbati, B., B. Biavati, and D. Palenzona, 1995. The genus *Bifidobacterium*. Pages 279-306 in The Genera of Lactic Acid Bacteria. B. J. B. Wood and W. H. Holzapfel (ed.). Blackie Academic and Professional Press, London, UK.

Tannock, G. W., A. Tilsala-Timisjarvi, S. Rodtong, J. Ng, K. Munro, and T. Alatossava, 1999. Identification of Lactobacillus isolates from th gastrointestinal tract, silage, and yoghurt by 16S-23S rRNA gene intergenic spacer region sequence comparisons. Appl. Envt. Microbiol. 65:4264-4267.

Temmerman, R., B. Pot, G. Huys, and J. Swings, 2003. Identification and antibiotic susceptibility of bacterial isolates from probiotic products. Int. J. Food Microbiol. 81:1-10.

Tortuero, F., 1973. Influence of the implantation of *Lactobacillus acidophilus* in chicks on the growth, feed conversion, malabsorption of fat syndrome and intestinal flora. Poult. Sci. 52:197-203.

Walter, J., C. Hertel, G. W. Tannock, A. Tilsala-Timisjarvi, S. Rodtong, D. M. Loach, L. Munro, and T. Alatossava, 2000. Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. Appl. Environ. Microbiol. 66:297-303.

Yeung, P. S. M., M. E. Sanders, C. L. Kitts, R. Cano, and P. S. Tong, 2002. Species-specific identification of commercial probiotic strains. J. Dairy Sci. 85:1039-1051.

Zulkifli, I., N. Abdullah, N. M. Azrin, and Y. W. Ho, 2000. Growth performance and immune response of two commercial broiler strains fed diets containing *Lactobacillus* cultures and oxytetracycline under heat stress conditions. British Poult. Sci. 41:593-597.